INVESTIGATIONS INTO THE SPECIFICITY AND MECHANISM OF ACTION OF THE ATTENUATED ADENOVIRUS dl1520 WITH PARTICULAR REFERENCE TO HEAD AND NECK CANCER

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Abstract

This thesis is concerned with investigating the selectivity, mechanisms of action and optimisation of delivery of the novel anti-cancer agent dl1520. Dl1520 is an attenuated adenovirus that has been genetically modified to have a selective cytolytic action against tumour cells deficient in function of the p53 tumour suppresser protein. This action follows on from a localised, productive infection of the virus. As defects in p53 gene function are known to occur in at least 60% of human solid malignancies this makes selective targeting of such cells an ideal strategy for anti-cancer gene therapy. The virus has been previously tested by direct intra-tumoural injection, in patients with recurrent SCC of head and neck and advanced tumours of ovary and GI tract and been found to have anti-tumour activity in a certain number of cases. In clinical trials the side effect profile of the virus has been generally favourable, with fewer adverse effects than would be expected with the standard systemic treatments currently available to treat these advanced tumours.

Recent publications have raised questions about the mechanism of action of the virus, with particular reference to its p53 selectivity. Some in-vitro experimental series have cast doubt on the selective action of dl1520 in p53 null tissues as detailed above. One research group has reported experimental findings that could be interpreted as suggesting that p53 is required for the virus to cause cell death, in direct contradiction to the accepted mode of action of the virus. Despite much evidence from laboratory based studies, there have not to date been sufficient data from earlier clinical trials to convincingly support the hypothesis of p53 selectivity. One reason for this is that samples taken from tumours treated with the virus have been relatively small and this has led to inadequate biopsy material for detailed histological analyses.

This thesis was conducted to report on experimental and clinical studies to further elucidate the p53 selective aspects of the action of dl1520 and how this relates to viral

spread and mechanism of cell death within tumours treated with the virus. Earlier experimental work has pointed to the therapeutic potential of dl1520 when combined with conventional cytotoxic agents. The study confirms that the virus can act in an additive fashion with standard chemotherapeutic agents in a tissue culture model. This effect is dependent on the p53 status of the cell line used when cells are exposed to the virus for a period sufficient for viral replication to take place. The virus under these circumstances causes greater cell death in p53 negative cells.

Using a murine xenograft model with cell lines matched for p53 status it is shown that the virus replicates and spreads preferentially in p53 negative cell lines, although it can exist and replicate within p53 competent tumours as well. Viral presence is maximal at 72hrs following administration. It is also demonstrated that the use of the local anaesthetic lignocaine increases the distribution of virus when used as a carrier medium. This effect is probably due to local vasodilatation. Using hyaluronidase as a carrier medium also increases viral distribution, an effect that is probably due to the liquefaction of ground substance in the tissue interstitium causing decreased resistance to the flow of the virus.

The clinical trial investigated for the first time the effect of a single intra-tumoural injection of the virus into oral squamous cell carcinoma in patients who have received no prior therapy. These patients were scheduled for potentially curative surgery 1-14 days following injection. A concomitant injection into adjacent normal tissue with dl1520 was also administered prior to surgical resection of the tumour and biopsy of the injected normal tissue. Data from this trial show that the virus can exist and replicate within human squamous cell carcinoma. Significantly more virus is detected in tumour tissue than normal tissue and we have shown that viral replication is probably at least partially p53 dependent. Injecting the virus into normal tissue samples causes no evidence of tissue necrosis, even though virus can be detected within some normal tissue samples. These studies suggest that the virus is selective for replication in tumour tissue as

opposed to normal tissue. This selectivity is probably dependent on the p53 status of these cells. In those cases where virus was detected in normal tissue biopsies there was immunohistochemical evidence of abnormality in the p53 protein in these samples. It is postulated that within some of these normal tissue biopsies there were abnormalities of function of the p53 protein that allowed the survival of the virus.

The mechanism of cell death within the injected samples was also investigated looking at levels of apoptosis within the specimens. Following the injection of virus into normal tissue at 24 hours there is a higher than expected level of apoptosis. It is probable that this apoptosis is caused by the adenovirus causing a p53 modulated cell death as would be predicted in normal, p53 competent tissue. This suggests the agent will have little capacity to replicate within normal tissues.

The circulating immune response to adenoviral injection was also investigated. It was demonstrated that at 24 hrs following injection there was a transient drop in CD4+ve lymphocytes in the peripheral blood. The mechanism of this drop is not clear at present nor whether there is any clinical sequelae related to it. No clinical effect on patients due to this drop was noted.

This thesis represents a body of work involving the novel anti-cancer agent dl1520. The results indicate that the virus does have a selective propensity for survival and replication within p53 deficient tumour cells. The virus can exist within normal cells and p53 competent tumour cells but to a lesser extent than in p53 competent cells. There is no evidence that the virus can harm normal tissues, even when directly injected into them. This is an important factor in considering the safety of a novel agent such as dl1520. The results of the above experiments show that the agent can safely be used for direct intra-tumoural injection for the treatment of squamous carcinoma of the head and neck with no apparent risk of collateral damage to surrounding structures.

CHAPTER ONE -

INTRODUCTION

Introduction

1.1 Background

Dl1520 is an attenuated adenovirus currently under investigation as a novel anti-cancer agent. The adenovirus has been modified in such a way that it will in theory destroy tumour tissue by direct cytolysis of tumour cells. This follows a localised productive infection within these tissues [1]. The virus has been modified so as to have a specific action against cells lacking function of the p53 tumour suppressor gene with little or no adverse effect on p53 competent tissues. As many human malignancies are characterised by mutation of the p53 gene or lack of function of the protein this selective targeting makes the dl1520 virus an attractive possibility as a therapeutic agent[2]. Human intra-oral SCC has a particularly strong association with p53 abnormalities [3, 4]. The specificity for p53 deficient cells is due to the inability of the virus to manufacture the E1B viral protein, which is necessary to bind and inactivate the p53 tumour suppresser protein [5]. Modifications to the genome of the virus itself have been made so that it does not express the E1B 55,000 gene product [6]. The virus contains a deletion between nucleotides 2496 and 3323 in the E1B region. There is also a C to T transition that generates a stop codon at codon 2022 so preventing expression of even a truncated protein [7].

The human adenovirus is a common human pathogen that in the wild type causes mild upper respiratory tract infections in humans [8]. Normally, adenovirus inoculation into a cell causes the cell to enter S-phase due to the action of the adenoviral E1A protein. This effectively switches on the cell cycle to create a suitable environment for viral replication [9]. Adenoviral infection however also triggers the activation of the tumour suppressor gene p53 leading to p53-modulated apoptosis via a pathway involving the retinoblastoma gene and $p14^{ARF}$ (see below for detailed description). This leads to the death of the host cell before viral replication can take place. In this way p53 is activated as part of the cellular protective mechanism against viral infection. The viral E1B protein

of adenovirus can block this p53-mediated effect so inhibiting apoptosis and allowing cell entry into S-phase, which is permissive for viral replication. Dl1520 lacks this E1B function and so is purported to be severely replication deficient in p53 competent cells.

Many human malignancies are characterised by dysfunctional p53 as part of the fundamental genomic change leading to or propagating malignancy [10] [11] [12]. The attenuated adenovirus dl1520 is thought to have selective anti-cancer activity, the selectivity being due to this lack of functional p53. The mechanism of cell death is cytolysis of cancer cells. It should cause little or no harm to normal tissues [12, 13]. This thesis is concerned with a series of experiments and a clinical trial to further investigate the action of dl1520 both in terms of laboratory based in-vitro and in-vivo models and also in a clinical trial involving the administration of the agent to human subjects. Factors affecting the optimal delivery of the virus have also been investigated.

1.2.1 Gene therapy of malignant disease

Cancer in humans is the third commonest cause of death in the Western world. Standard treatments include surgery and radiotherapy to treat localised disease, and chemotherapy [14]. Chemotherapy can be administered systemically to try to destroy metastatic lesions or to address a recurrent tumour that cannot be surgically removed. Surgery and radiotherapy can be used with curative intent or for palliation whilst chemotherapy is most commonly used as an adjuvant to other treatments or as salvage treatment once other therapies have failed. Surgery and/ or radiotherapy can be very effective first line therapies especially for early cancers where the disease is localised to one anatomical area [15]. Once a tumour recurs following such treatment or for disease where there is spread either to lymph glands or distal metastases these treatments can usually only offer palliation. Chemotherapy may be indicated to try to deliver a systemic therapy throughout the body to control disease. Squamous cell carcinoma (SCC) of the head and neck is such a form of cancer, which has only a limited response to chemotherapy once

disease is recurrent or metastatic [16].

For most solid tumours attempts to control widespread or recurrent disease are palliative only and any response is partial and short-lived. If for example a patient has a recurrence of disease following surgical treatment for squamous tumour of the head and neck they have a life expectancy of only three months. This is not significantly improved with chemotherapy although there have been some promising recent trials [17, 18].

Most chemotherapy agents act to selectively kill cancer cells due to specific genetic differences that these cells possess as compared to normal cells. One example is the commonly used DNA damaging agent cisplatin that relies on the function of the p53 tumour suppressor gene to cause apoptosis in cancer cells following exposure to the drug. Cancer cells that are resistant to cisplatin often have reduced function of the p53 protein [19]. The action of cisplatin therefore relies on the function of cell cycle regulatory proteins such as p53, which are required to couple the activity of DNA damaging agents to the cell death apoptotic pathway [20]. Another example is the anti-tumour agent Camptothecin that targets DNA Topoisomerase I. This allows a selective toxicity against tumour cells that have a high turnover but the therapeutic index is often narrow. This is because all cells with a relatively high turnover are targeted along with cancer cells leading to side effects such as hair loss, immunosupression and gastro-intestinal upset. The combination of limited clinical effect and poor side effect profile of chemotherapy for most recurrent or metastatic cancer mean that alternative treatments for such disease are required that would have greater specificity for tumour cells both locally recurrent and metastatic.

One group of treatments that can potentially address these problems is gene therapy. Gene therapy can be defined as follows: 'Somatic gene therapy research is considered to include the use of genetically modified organisms and/ or modified nucleic acids or modified human somatic cells for potential research or for other research purposes in

human subjects.'

Gene therapy for cancer entails trying to correct the genetic fault within cancer cells at the molecular level either leading to the death of these cells or transforming the cells out of their cancerous state [21]. Common genetic problems in cancer cells include lesions of dominant oncogenes or tumour suppressor genes [22]. Gene therapy for cancer aims to alter these genetic faults. This can either lead to destruction of tumour cells or transformation of a malignant cell back to a normal phenotype. It is hoped that this might lead to improved therapies for cancer. There are several potential strategies by which this can be achieved [23]:

1.2.2 1- Strategies for gene therapy

1. A functional gene can be inserted to override the effect of a dysfunctional gene e.g. a tumour suppressor gene. The p53 gene for example is mutated in many human cancers and this loss of p53 function is thought to be a fundamental change leading to malignant change. By inserting a functional copy of the p53 gene into cancer cells this could lead to the cell transforming to a non-malignant type [24-26].

2. Homologous recombination is the technique whereby exogenous DNA is integrated into a host cell genome [27]. This allows for subtle modifications of point mutations or insertions with the potential for specific gene repair [28, 29]. It is possible that by identifying specific target genes involved in carcinogenesis and replacing these with the functionally normal DNA segment homologous recombination could be used as a therapeutic strategy [30].

3. A suicide gene can be added selectively to tumour cells that codes for an enzyme which converts a harmless pro-drug into a cytotoxic agent. This would lead to a local accumulation of the cytotoxic agent only in the cancer cells targeted by the gene therapy

agent [31]. Using this technique there is also the bystander effect whereby cells not directly targeted with a gene therapy agent are nevertheless killed due to local cytotoxic activity [32, 33]. Bystander effect can also be seen in strategies that rely on the host immune system to destroy tumour cells. For example tumour cells can be genetically modified with the herpes simplex virus and then targeted with Gancyclovir. This suicide gene therapy has been shown in mice to induce an immunologically mediated bystander effect, which can be maximised by prior immunisation of the mice against tumour cells [34] [35]

4. Anti-sense gene therapy involves the transcription of a mRNA product that is transcribed in the opposite direction of a target oncogene. This mRNA segment would be able to bind to the target oncogene effectively blocking the production of the oncogenic protein [36]

5. Certain small inhibitory RNA molecules are known to suppress the expression of specific proteins [37]. These have been used experimentally to reduce the expression of certain key proteins important in DNA double strand break repair, potentially rendering target tumours more radio and chemo-sensitive [38].

6. Anti angiogenic therapies target the blood supply of tumours using anti-angiogenic factors. In a mouse model it has been shown that intra-peritoneal delivery of anti-angiogenic factors can inhibit breast tumour formation [39].

7. Protective gene therapy involves the potential use of gene therapy to insert genes coding for protective proteins which could allow for higher than normal doses of cytotoxic drugs to be administered. One such example is the Multi Drug Resistance protein which is known to protect normal tissues from certain anti-cancer drugs [40].

8. Immunotherapy strategies can be used which are particularly suited to treat

disseminated disease, due to the systemic nature of the immune system. Strategies can involve activating an antibody or cell mediated immune response against specific tumour antigens [41]. Current research strategies in this field include immunisation with tumour cell vaccines; immunisation with tumour peptides or DNA encoding tumour peptides [42]. It is also possible to attempt to enhance immunity by blocking inhibitory factors acting on T-cells.

Before gene therapy can be effective several steps must be taken. The target gene must be identified and the desired DNA produced. The genetic material must then be inserted in to the desired cells either within the host directly (in-vivo) or following manipulation of the target cells outwith the host (ex-vivo). Vectors for delivering gene therapy are crucial to the success of these strategies.

1.2.2.2 Vectors in gene therapy

Many different strategies to devise suitable gene therapy vectors have been attempted [23, 43]: Viral vectors are by far the most efficient and widely used techniques for gene transfer involve the use of viral vectors. Many attempts have been made to utilise the ability of viruses to integrate into human cells in the gene therapy treatment of cancer [44]. The most commonly used viruses include retroviruses and adenoviruses. Retroviruses integrate their DNA into the target cells thus ensuring any progeny cells maintain the genetic manipulation. Adenoviruses are capable of carrying large amounts of genetic information and can enter human cells readily [45, 46].

Non-viral vector techniques can also be used for gene therapy. Naked DNA can be directly inserted, a process which can be facilitated using electroporation; gene gun; ultrasound and hydrodynamic pressure [47]. It is also possible to construct vectors consisting entirely of chromosomal material which can integrate into the genome [48]. These areas can Potentially act as independent domains within the genome. All these

non-viral vectors have potential advantages in terms of safety and likelihood of eliciting an immune response, but suffer from relatively low transfection efficiency [49].

1.2.2.3 - Targeting diseased cells

For a gene therapy strategy to be successful in cancer, it is necessary to target cancer cells involved in the disease process preferentially whilst minimising damage to healthy tissues. Malignant cells can be of many different types and can exist in all areas of the body. Effective targeting of these cells is therefore desirable to maximise the therapeutic process and minimise the risk of germ line transduction. This is clearly more important for systemic gene therapy strategies as opposed to direct intra-tumoural delivery. There are many potential strategies for targeting under investigation [50].

1.2.2.3.1 - Targeting Gene Delivery

It is possible to target delivery of a gene therapy vector to a specific area. An ideal vector for gene delivery should be targeted, protected from degradation and safe for systemic delivery. It should allow for systemmic administration and then go on to accumulate in the target tissue. Factors such as binding specificity; size of particle; charge of particle and route of administration will all affect the bio-distribution of a given vector [51].

1.2.2.3.2 - Non-replicative viral vectors

1. Conjugate based targeting allows viral vectors to be targeted by the use of bifunctional bridging agents which can attach to both the virus in question and a given target tumour cell type. For example bifunctional crosslinkers that recognise fibroblast growth factor and epidermal growth factor receptors (both over expressed in many tumour types) have been developed [52].

2. Genetic targeting describes a process whereby a vector virus is modified to bind preferentially to a selective target cell. This has been demonstrated where ecotropic murine leukemia virus was modified by the addition of a ligand to bind to EGFR [53]. It is also possible to restrict the ability of a virus to bind to its natural receptor and therefore re-direct it to a chosen target cell [54].

1.2.2.3.3 - Replicative viral vectors

Replication competent virus strategies can potentially allow for much greater release of gene product at a target tissue. The risk of damage to surrounding tissue is however also potentially less controlled than in replication deficient strategies. Targeting of these agents is therefore important.

1. Partial de-activation of virus can allow for a virus to selectively replicate only within target tissues. dl1520 adenovirus is an example of this strategy and is selective against p53 deficient tissues due to an E1B gene deletion. This strategy is described in greater detail below.

2) Control of transcription of viral genes required for replication can be utilised by replacing the native viral promotors with other promotors activated by conditions specific to the target tumour cells. An adenovirus has been thus modified to be activated in the presence of hypoxia and oestrogen as a strategy to target breast cancer [55].

3) It is possible to modify the signaling pathway in tumour cells to restrict replication to certain tissue types. One example is the modification of the adenovirus that has been modified to replicate selectively in cells with PSA (prostate specific antigen), a protein strongly associated with prostate cancer [56].

1.2.2.3.4 - Non-viral vectors

Non-viral vectors are attractive targets for gene therapy as they are less immunogenic, have less risk of side effects and are easy to administer. Ligands to specific receptors can be used to target these agents to specific tumour cell types [57]. One example of this strategy is the addition of an epidermal growth factor targeted polyethylenime system that might be used to target breast or prostate cancer where these receptors are over expressed.

1.2.2.3.5 - Targeted bacterial vehicles

Bacteria are attractive as vehicles for gene therapy due to their large genome and the potential to control their replication with anti-biotics. Several bacteria are under investigation as they can be targeted to the relatively anaerobic conditions found in the centre of tumours. The anaerobic Clostridium sporogens, transfected with cytosine deaminase, has been used as a suicide gene strategy combined with the pro-drug 5-fluorocytosine [58]

1.2.2.3.6 - Targeted gene transcription

Targeting gene transcription to specific cells rather that delivery is another strategy currently under investigation. This is potentially more selective than gene delivery targeting as most receptor targets are not totally specific to tumour cell types.

1.Tissue specific transcriptional regulatory sequences can be utilised such as the glial fibrillary acidic protein promoter in glioma cells[50].

2. Promotors specifically activated by conditions prevalent in tumours can be utilised. One example is hypoxia related gene expression causing hypoxia related gene expression [59].

3. Anti-sense oligonucleotides can be used to specifically target mRNA of pathogenic genes. In theory this allows for very precise targeting of pathogenic genes, but this strategy is at an early stage of development [60]

4. DNAzymes are single stranded oligodeo-xylnucleotides capable of cleaving mRNA. These can be used to specifically target pathological gene products. As an example, the vascular endothelial growth factor receptor is an important target for anti-angiogenic gene therapy. This can be targeted by a specific DNAzyme which has been shown to digest mRNA substrates of this receptor, leading to reduced tumour growth in a murine model [61].

1.2.2.3.7 - Cells used as carriers

It is possible to utilise various cell types to transport gene therapy agents following systemic delivery. Clearly these cells need to localise to target tumours. [62].

1. Macrophages can be used to transport transcriptionally targeted vectors to increase anti-tumour activity [63].

2. T cells can be utilised as carries due to their propensity to extravasate in response to specific signals. This strategy has been used in T-cells expressing a chimeric T-cell receptor against CEA, expressed in colorectal tumours [64].

3. Tumour cells, either autologous or allogenic, can in theory be used as vector carriers as it has been noted that tumour cells bind preferentially to other tumour cells of the same type [65].

4. Stem cells also have potential as carriers of gene therapy vectors base don the fact that

these cells can differentiate into different cell lines. This would remove the need for harvest of the various cell types described above, as they could be grown direct from stem cells. For example it has been shown that stem cells from bone marrow can differentiate into endothelial cells [66].

1.2.2.4 - In-vivo and ex-vivo techniques

Gene therapy can be further divided into in vivo or ex vivo techniques. *In vivo* gene therapy involves genetically modifying cells within the body by introducing a therapeutic gene (DNA or viral vectors) via direct administration into the patient through direct tumoural, intra-venous, intra-peritoneal, intra-muscular or intra-arterial injections. E*x vivo* gene therapy involves genetically modifying cells that have been removed from the body and culturing them to allow for expansion. This strategy lends itself to techniques involving cells of the hematopoietic system [67] [68]. The cells are then later reintroduced to the host. Both methods seek to genetically modify cells in a manner that will allow for prolonged gene expression.

1.2.3 Clinical trials in gene therapy

The first clinical trial showing proof of principle of gene therapy for malignant disease was reported in 1996 when Roth et al successfully integrated functional p53 DNA via an adenoviral vector into recurrent non-small cell lung cancers by direct intra-tumoural injection [69]. Subsequently evidence of vector-p53 sequences was demonstrated within the tumour along with evidence of increased apoptosis. The same group have conducted phase I and II trials using direct intra-tumoural injection of Ad-53, combined with both chemotherapy and radiotherapy. As with many clinical trials, problems with delivery of the virus have limited the success of this approach [70]. Prostate cancer has also been targeted with gene therapy. A phase I trial using intra-prostatically administered interleukin 2 encased in a DNA-lipid complex in patients with advanced prostate cancer

showed an increased T-cell infiltration in prostate tissue and a decrease in expression of prostate specific antigen [71].

Despite this early success and numerous clinical trials there remain many problems with gene therapy as a treatment for cancer. Difficulties have so far been encountered with delivery systems, as targeting tumour cells selectively is problematic. To successfully deal with systemic disease the ideal delivery vehicle would allow repeated systemic treatments with a selective uptake of the desired gene into tumour cells. Non-viral delivery systems are relatively inefficient at delivering genetic material and viral vectors tend to promote a vigorous immune response leading to rapid clearance of a proposed vector if it is delivered more than once [46, 72]. Direct intra-tumoural injection is possible and may elicit less of an immune response but will not deal with inaccessible, blood borne or microscopic disease.

There are safety issues concerning the administration of gene therapy, particularly associated with viral vectors. Toxic viraemia; the effect of virus on the liver or other organs; passage of virus around the community and local tissue reactions are all possible complications with these approaches.

1.3.1 Viral gene therapy using targeted cytolysis

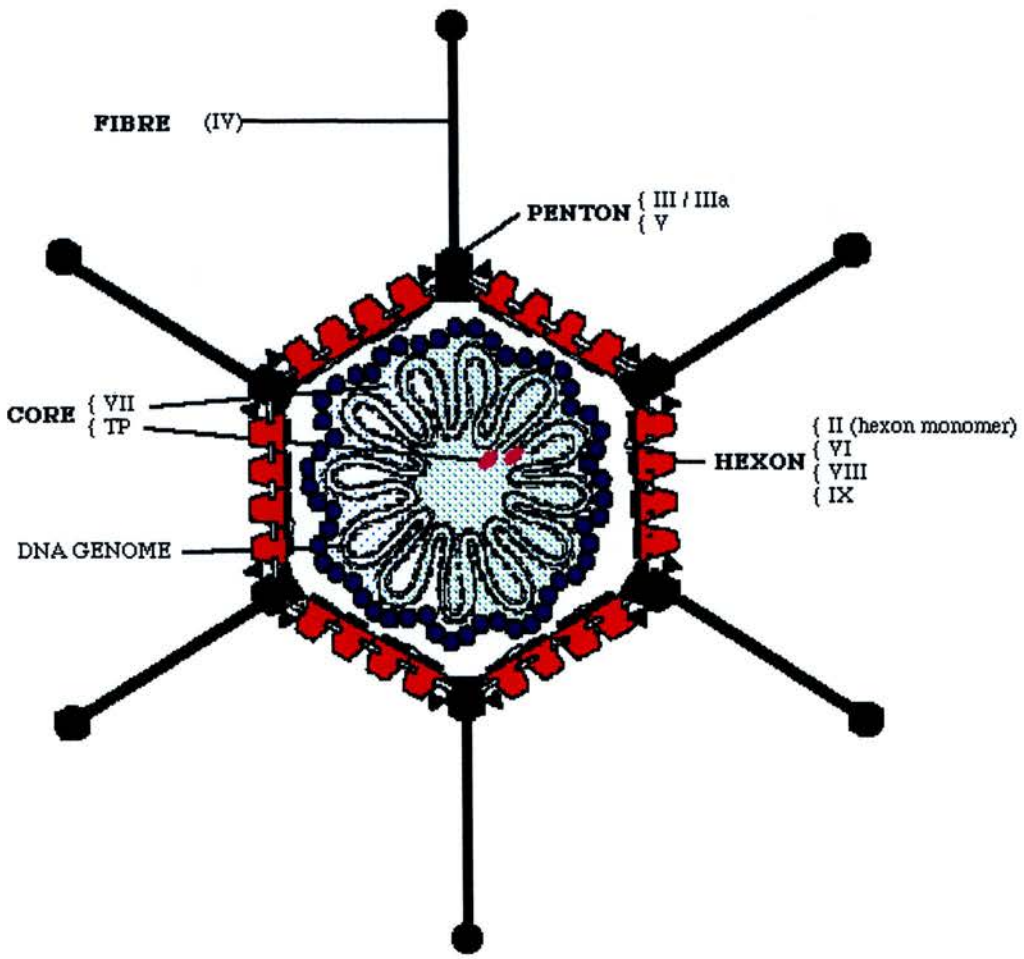
Another possible use of the technology of gene therapy is to utilise the direct cytotoxic effect of a virus targeted to tumour cells. Many viruses can infect cells and after a period of replication lyse these cells to release further virus particles which can go on to infect surrounding cells leading to a productive viral infection [73]. It has been known for almost a century that certain viral infections or rabies vaccination could occasionally lead to remission in malignant disease. Many researchers have thought of using oncolytic viruses in patients with advanced cancer and the first successful use of an adenovirus was reported in 1956 when patients with advanced cervical cancer were treated with

direct intra-tumoural injection with some favourable responses [74].

Modern techniques allow for high levels of viral titre to be manufactured and there has been a recent upsurge in interest in this form of therapy. The idea of a virus targeting tumour cells directly is attractive for many reasons. It is known that after a small number of tumour cells are killed by viral replication and cytolysis in this way there is a significant bystander effect [6]. If a virus is directly lytic to tumour cells there is no need to instil foreign genetic material into human cells. If a virus can somehow be made to target tumour cells only it should be possible to deliver a highly selective anti-tumour agent which might be delivered systemically to treat disseminated or microscopic disease. The side effect profile of such a treatment should be acceptable when compared to other standard treatment regimes.

An ideal candidate for such a virus would be able to replicate within cancer cells; should cause only mild symptoms in the host and be non-integrating into human DNA [75]. Adeno-viruses are common human pathogens that fulfil the above criteria. They are currently favoured for many gene therapy trials as a vector as they can store a lot of DNA and integrate into a wide range of host cells [45]. They can also act in a directly cytolytic fashion following cellular inoculation. The adenovirus is a common pathogen that leads to upper airway infection and symptoms of coryza or common cold (Fig 1-1). The wild-type virus typically inoculates into cells and after a period of replication goes on to lyse the infected cell releasing further virus particles which can go on to infect further cells. One problem with using adenovirus as a vector is that a large proportion of the population have been exposed to the pathogen and will have mounted an immune response, meaning the body will react vigorously to a repeated viral infection [76].

Figure 1.1– adenovirus structure (University of Leicester Department of Microbiology and Immunology website www-micro.msb.le.ac.uk/)



The **penton fibres** consist of a slender shaft with a globular head. They are involved in the process of attachment of the virus particle to the host cell.

1.3.2 Mechanism of cell lysis

The virus initially binds to specific cell surface receptors before being internalised by endocytosis [8, 73, 77, 78]. Following adenoviral infection of cells a series of metabolic defects appears within the cell notably reduction in RNA, DNA and protein synthesis coupled with a loss of structural integrity of the cytoskeleton of the cell leading to lytic cell death. A major effect of this loss of host cell protein synthesis is to directly weaken the cytoskeleton so as to increase cell lysis with subsequent viral spread rather than simply to direct metabolic activity toward viral product synthesis [79]. This effect takes approximately 36 hours [80]. Thus if cytolysis is reduced there is a dramatic reduction of the ability of the adenovirus particles to spread.

1.3.3 dl1520

To be effective as a viral therapy treatment a virus would need to be selective for either infection of or replication within tumour cells. This would mean that that normal healthy tissue would not be affected along with any tumour cells. Such selectivity can be accomplished by making cell entry selective; by making transcription of certain genes only possible in tumour cells or by removing genetic material necessary for replication in normal cells but which are not required for replication in tumour cells. There are several examples of this last type of therapy under investigation. A replication conditional herpes simplex virus has been used in the treatment of brain tumours [81]. An adenovirus selective for replication in cells carrying the alpha-fetoprotein promoter has been used to target hepatocellular carcinoma in nude mice [82]. The dl1520 adenovirus falls into this group of treatment strategies.

The attenuated adenovirus dl1520 was initially developed as a research tool to investigate the requirements of various viral proteins for transformation of rodent cells [5]. It is postulated that this virus should be selective for replication in cells deficient in

the tumour suppressor p53 protein and that this selectivity could lead the virus to be used as an anti-cancer agent [6]. Dl1520 has been constructed based on the wild-type type 5 human adenovirus, the wild type version of which is known to cause only mild, self limiting illness consisting of fever, headache and nausea. The side effect profile of any modified virus based on an adenovirus would be expected to be favourable as the wild-type virus only causes mild symptoms under normal circumstances.

1.3.4 Mechanism of action of dl1520

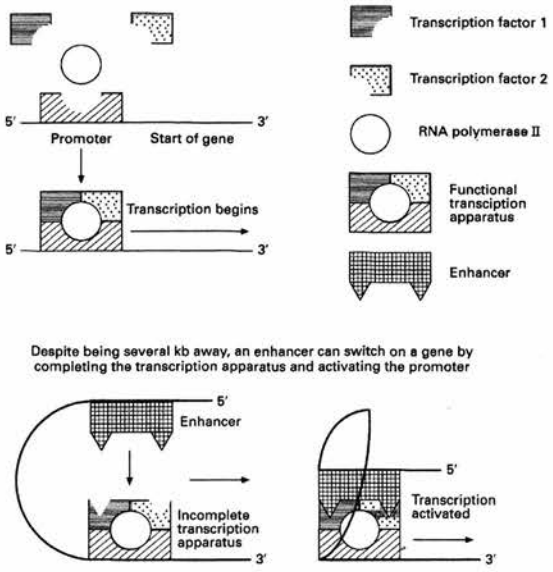
The dl1520 adenovirus is a gene deleted group 5 adenovirus deficient in the viral E1B protein. It has also been found that the virus is partially deficient in the expression of E1A mRNA and protein. It is known that viral replication activates host cell p53 via the E1A viral early protein. This involves a signalling pathway involving both the retinoblastoma protein and the tumour suppressor p19 ^{ARF}. [9]. E1A binds to a product of the retinoblastoma gene. The retinoblastoma susceptibility gene product, pRb, can interact with the E2F meaning in effect a loss of Rb gene function. This in turn leads to the induction of p53, via an increase in the expression of p14^{ARF}, which prevents MDM2 controlled degradation of p53 [83] [84]. It is thought that the E1B protein acts to de-activate p53 by forming a complex with p53, involving the E4ORF6 viral protein [85].

The virus has been constructed to contain an 827-base pair deletion in the E1B region and a point mutation at codon 2022. This prevents expression of both normal and truncated forms of the E1B protein. This E1B protein, along with E4Orf6 protein, can normally form a complex with the human tumour suppressor p53 protein, leading to p53 inactivation. It appears that this inactivation happens through two distinct mechanisms:

1.The E1B protein is thought to bind to an area of the p53 protein in the N-terminus at amino acid residues 22 and 23. This area is known to be important for translational upregulation of the protein and is also where the MDM protein binds to p53 to cause

suppression of activity of the protein [12, 86]. Transcriptional upregulation is one of the processes by which the production of a specific protein can be increased. This is achieved by a DNA control element frequently found 5' to the start site of a gene. This element binds to a specific transcription factor that enhances expression of the gene[87]. It seems therefore that the E1B protein bind to specific amino acids within the N-terminal domain which are important for the positive regulation of transcription of the protein (see figure 1.2).

Figure 1.2 – regulation of transcription (from Human Genetics, Chapter 2 pg 22. Gardner A. (Pub Arnold))



2. The E1B-55K protein possesses an intrinsic domain that represses transcriptional activity. This area targets p53 specific promotors via a direct action on p53 that is bound to DNA [88].

This inactivation of p53 is necessary for viral replication following adenoviral infection. Following viral infection the E1A early viral product normally induces the host cell to enter the S-phase of the cell cycle to encourage proliferation of cells [89]. This in turn activates the p53 tumour suppressor gene and it is thought that the pathway of this activation involves the retinoblastoma protein and the tumour suppresser protein $p19^{ARF}$ [9]. This normally leads to controlled cell death by apoptosis so preventing viral replication and containing any viral infection. E1B viral protein inactivates p53 via the binding detailed above, allowing viral replication and eventual cytolysis rather that apoptosis which would curtail intra-cellular viral replication and subsequent spread.

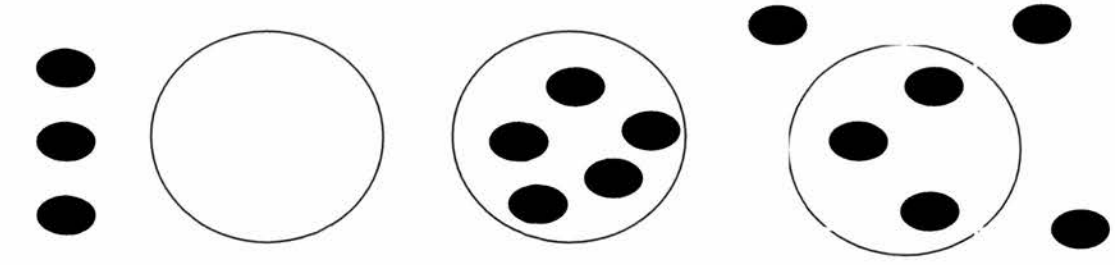
It was originally hypothesised by Dr Frank McCormick that a virus deficient in function or expression of the E1B protein would have restricted capacity to replicate in normal cells but in p53 deficient cancer cells it would be replication competent [5]. If dl1520 infects a cell with a functional p53 the virus cannot block the action of p53 and so the cell would apoptose, containing any infection and preventing viral replication. In the case of a cell deficient in p53 function this would not occur and the dl1520 virus could replicate prior to causing cell death by cytolysis and causing a localised productive infection (see figure 1.3). As p53 mutation and dysfunction is present in at least 60% of human solid malignancy, including SCC of the head and neck, dl1520 is able to selectively infect and destroy many tumour cell lines [90, 91]. The virus should not cause significant harm to healthy tissue with a functional p53. A significant body of work has been undertaken both in vivo and in vitro to determine if the dl1520 virus can indeed function to cause p53 specific cytolysis in this manner.

Targeted cytolysis via replication competent viruses has many potential attractions as a highly targeted, potentially systemic treatment with the potential to deal with micro-metastatic disease. The capacity of the virus to replicate means that effective amplification of the input dose can take place [92]. There are potential disadvantages however to this approach. Viruses can trigger inflammatory and immune responses

which can be unpredictable [93]. Experimental data utilizing these agents has been derived from studies involving nude mice, where the host immune system plays no part in fighting a viral infection. All viruses are potentially harmful, even though the commonly used adenovirus has few significant symptoms in healthy individuals. Candidates for gene therapy are often immunosuppressed and may succumb to a viral infection triggered by a gene therapy vector, as evidenced by a subject who died in a clinical trial utilising adenoviral gene therapy to treat ornithine transcarbamylase (OTC) deficiency react more strongly to viral infection [94]. Attenuated adenovirae such as dl1520 have also lost other functions apart from that allowing for targeting of tumour cells. Dl1520 is known to be missing vital E1B functions related to mRNA transport and so will be attenuated in virulence even in p53 null cell lines [95].

Figure 1.3 - Mechanism of Action of dl1520.

WILD TYPE ADENOVIRUS IN NORMAL P53 COMPETENT CELL

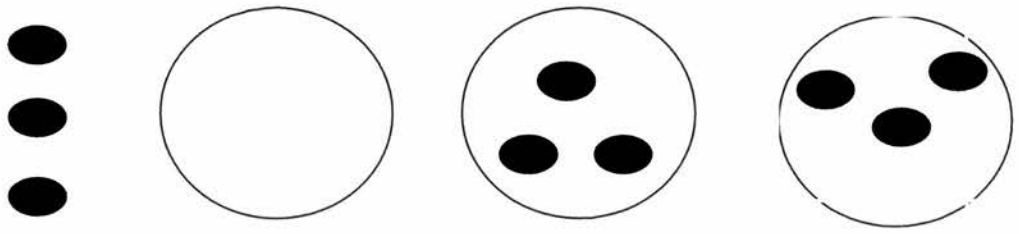


Virus innoculates into cell

Viral replication triggers p53, which is blocked by E1B

Viral replication leading to cytolysis and viral spread

dl1520 ADENOVIRUS IN NORMAL P53 COMPETENT CELL

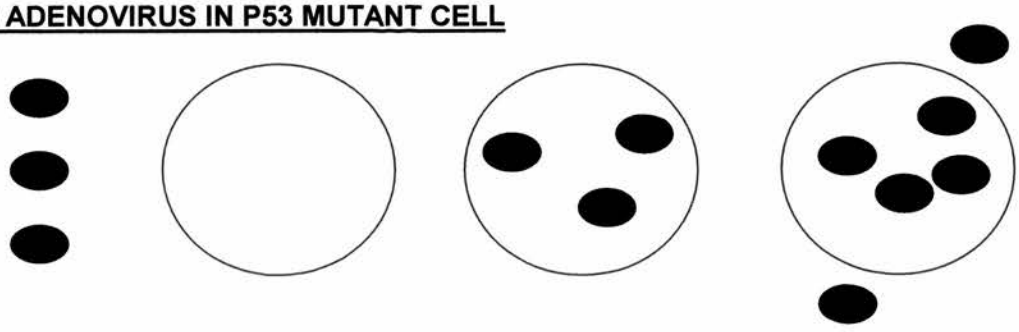


Virus inoculates into cell

Viral replication triggers p53, which is not blocked by E1B

Cell undergoes apoptosis preventing spread of virus

dl1520 ADENOVIRUS IN P53 MUTANT CELL



Virus innoculates into cell

Viral replication with no p53, so apoptosis is not triggered

Viral replication leading to cytolysis and viral spread

1.4 P53

The p53 protein is a transcription factor now widely recognised as being of paramount importance in the development of many examples of human carcinogenesis. Lane et al identified the protein in 1983 and since this time it has become clear that it has an important tumour protector role [96]. Mutations or lack of function of p53 are known to contribute to the formation of many if not most solid malignancies in man [13]. This is particularly so in the case of intra-oral SCC in humans [90, 97].

When functioning normally p53 acts as a guardian of the genomic integrity of the cell. In healthy cells the protein is expressed but has a very short half-life and is undetectable by conventional immunohistochemical means. If the cell sustains an insult such as radiation damage that could lead to DNA damage p53 is activated. The main trigger to this activation is a double strand break in the DNA. Viral infection also acts to trigger activation of p53 via the viral E1A gene product [9]. P53 binds to promoter sites of various genes notably p21 WAF1, gadd45, mdm2, bax, thrombospondin 1 and cyclin-G all of which are involved in control of the cell cycle [11, 12]. Since its discovery there has been extensive research on the function of p53. It is known to have a significant role in controlling the cell cycle and to function as a protective element against carcinogenic cell damage. Conversely any loss of p53 function is thought to lead to cancer formation in many instances. For example in squamous carcinoma of the head and neck mutation of the p53 gene has long been known to be a key feature of most of these tumours [98]. This is particularly so in patients with a known history of tobacco and alcohol exposure, the main risk factors for the disease in the West [91]. It is also known that if the cells surrounding an excised tumour have p53 mutations the cancer is more likely to recur than if the resection margins are clear of p53 abnormalities [90]

The protein normally exists as a 53kDalton protein of 393 amino acids, which can be divided into four structurally separate domains. Following translation the activity of p53

is thought to be controlled largely by phosphorylation of several well-defined sites within the N- and C- termini of the protein [99]. Mutant or dysfunctional p53 is a common finding in more than 50% of human cancers. Several thousand distinct mutations have been recorded and are catalogued in several data bases available on the internet (for example the International Agency for Research on Cancer www.iarc.fr/p53/). The mutation is commonly a missense mutation that leads to the production of an abnormal protein. This protein then collects in affected cells leading to increased detection of p53 by immunohistochemical analysis but reduced p53 function [12]. Reduced or absent function of p53 seems to be implicated in a loss of the cells capacity to protect itself from many outside effects which if unchecked can lead to or encourage carcinogenesis. Dysfunctional p53 has been found in 60% solid human cancers and seems to be more common in tumours demonstrating chemo or radio resistance [100, 101].

Normally the p53 protein functions to protect the cell from damage and to prevent any error within the cell being propagated to future generations [13]. P53 can act to regulate the expression of proteins involved in negative regulation of cell cycle control and can also reduce transcription of genes responsible for up regulation of cell turnover [102]. There are several mechanisms by which this is thought to happen.

P53 can cause a cell cycle arrest at G1 or can lead to a cell undergoing apoptosis. These effects act to protect the cell from undergoing cell division under adverse circumstances so that potentially carcinogenic effects are not propagated. In certain circumstances p53 will trigger a cell to apoptose so as to prevent a harmful effect continuing. p53 is also activated in response to viral infection in an attempt to prevent intra-cellular viral replication. This effect is triggered by the early adenoviral product E1A but inhibited by the adenovirus E1B55Kd protein in the wild-type virus [89].

The half-life of normal p53 is less than 20 minutes and normally the protein exists at a

low concentration. There is also evidence that p53 can exist in a latent form prior to activation [11]. Several agents are known to stimulate p53 to cause either cell cycle arrest or apoptosis. Any DNA damage in a cell signalled by a single strand break or a increase in levels of DNA repair elements leads to an increase in p53 levels and an increase in the half life of the protein [2]. Different types and intensity of radiation damage can lead to varying levels of p53 expression. Hypoxia or a deficiency of ribonucleoside triphosphate can all lead to p53 activation as a means of halting cell division under unfavourable conditions [103].

Following activation and transcription p53 can act to prevent cell duplication in several ways. One down stream gene is p21 [104, 105] and once activated binds to several cell cycle kinase inhibitors, which can act to prevent the activity of DNA polymerase. Experiments with mice which are deficient in p21 have shown a deficient capacity to produce a G1 arrest in response to DNA damage which suggests that p21 has a role in this p53 mediated effect [106]. There is also evidence of a p53 dependent G2 arrest [107] where by p53 can prevent a cell prematurely entering S phase.

P53 is therefore a protein that has been found to be of fundamental importance in human malignant disease and carcinogenesis. The protein normally exists in low quantities or in an inactive state but in response to genetic damage or cellular inoculation by a virus the p53 can respond:

1. To act directly as an anti-mutator

2. To cause cell cycle arrest

3. To cause cell death by apoptosis

If there is a mutation in the p53 gene and these various protective pathways are disabled

any carcinogenic insult can be propagated potentially leading to tumerogenesis and cancer formation. The importance of p53 in clinical terms is heightened by the fact that many anti-cancer agents work via p53 dependent means so that p53 deficient tumour cells are often resistant to chemotherapeutic agents as well as radiotherapy [108].

1.5 P21 (WAF1)

Two research teams discovered the p21 protein simultaneously. Following on from the realisation of the importance of p53 in controlling the cell cycle it was found that the induction of a gene WAF1 was associated with wild type but not mutant p53 expression. cDNA from this site was also found to suppress many tumour cell lines [104, 109]. The protein product of this gene, p21, was found to tightly bind and inactivate cyclin dependent kinases (Cdk's) [110]. Cdk's are known to regulate the cell cycle via phosphorylation of the retinoblastoma gene (Rb) and thus p21 can act as a regulator of the cell cycle. P21/ WAF1 is induced by p53 and has been shown to accumulate and be functional in cells undergoing p53 mediated G1 arrest and apoptosis but not in cells undergoing p53 independent G1 arrest and apoptosis. P21 activation leads to a decrease in Cdk activity and is a key effector of p53 related G1 arrest and apoptosis and it has been shown that this function is required for p53 mediated G1 arrest [111]. P21 can also act as a direct repressor of tumour cell growth and tumourigenicity [112].

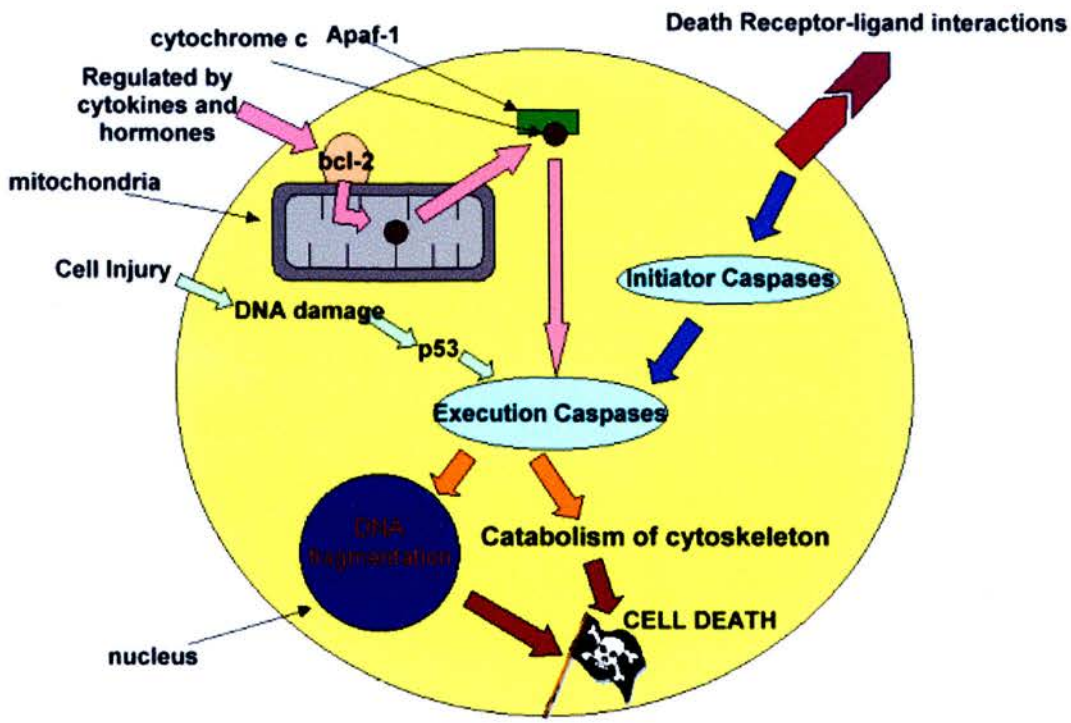
1.6 Apoptosis

Apoptosis refers to the pathway of programmed cell death that allows cells to be destroyed in a controlled manner [113]. The process involves a characteristic pattern of cytoplasmic changes, chromatin condensation and DNA fragmentation described by Wylie in 1980 [114]. This capacity of a cell to 'self-destruct' involves the complex integration of multiple control mechanisms [115, 116]. Apoptosis is involved in normal development of organs. It is also of fundamental importance as a protective mechanism

against cancer and viral infections and it is this that is of importance in the field of viral therapy. Various pathways can lead to the triggering of apoptosis but the common final pathway involves the influx of calcium ions and the activation of an endogenous endonuclease. This enzyme causes the break up of the nucleus with a characteristic pattern of nuclear degeneration into apoptotic bodies [117] (see figure 1.4).

In human squamous carcinoma of the head and neck it is thought that the HSP70 protein can act to prevent apoptosis thereby encouraging cell proliferation [118]. As has been stated above the adenoviral E1B protein is known to be a potent inhibitor of apoptosis, an action which is thought to be due to the protein blocking the action of the cell death promoting Bax protein [119]. Apoptosis can be thought of as a cell's last line of defence against a carcinogenic insult as the process allows the cell to die in a controlled manner as a response to carcinogenic insult. Factors that act to block apoptosis take away this final possibility to prevent propagation of any such carcinogenic effect. Loss of a cells ability to undergo apoptosis is known to be important in SCC of the head and neck [120].

Figure 1.4 - Mechanism of apoptosis (from Edinburgh University, Department of Pathology www.portfolio.mvm.ed.ac.uk/)



1.7 Control of the cell cycle

Before a cell can undergo mitosis to create two daughter cells many steps have to be achieved which are necessarily under tight control. p53 acts as a key regulator of this turnover as described above. It is known that cyclin dependent kinases (cdk) act to drive this cell cycle and they in turn are under the control of various G1 cyclins [121]. The retinoblastoma tumour suppressor gene is one of the key regulators of the cell cycle through its gene product pRB [122]. Before a cell enters the S-phase of the cell cycle to allow mitosis, the retinoblastoma gene can be activated by phosphorylation. This can be stimulated following E1A viral protein expression (for review see [123]). Various

growth factors act throughout the G1 gap phase to drive the cell to enter the DNA synthesis S-phase, eventually leading to mitosis, M-phase. Many regulatory mechanisms can act to prevent this cell cycle under adverse conditions [124]. Defects in the regulation of proteins involved in regulation of the cell cycle are implicated in many human malignancies, and this is known to be important in SCC of the head and neck [125].

1.8 Controversies involving the mechanism of action of dl1520

This synopsis of the action of dl1502 as a p53 specific anti-cancer agent has recently been called into question. A paper by Hall et al gave results from tissue culture experiments involving both wild type adenovirus and dl1520 [126]. Using a cytopathic effect assay on cell lines with either normal, mutant or absent p53 they assayed the replication of each virus. They showed that tumour cell lines expressing both functional and mutant p53 allowed replication of the dl1520 virus but that replication was greatest in cells with functional p53. Results using the supernatant of fluid from these experiments showed that the lack of cytopathic effect in p53 mutant and null cell lines was not due to lack of replicating virus. They suggest that wild-type p53 may in fact be required for cell death following adenoviral infection. Further experiments showed that cell death associated with wild-type p53 was not primarily due to apoptosis, implying that p53 is a necessary mediator of the cytopathic effect of wt adenovirus and dl1520. They conclude that adenovirus of both wt and E1B deficient can replicate within cells of varying p53 status, but functional p53 may be required for a productive, cytopathic adenoviral infection.

This is an entirely different interpretation of the action of the virus than had been suggested previously and is in direct contrast to previous findings that demonstrated that cells with a mutant p53 were more sensitive to the effect of dl1520. One weakness of the paper by Hall et al is that matched cell lines were not used and results were taken after a

relatively short incubation with the virus. Cells, which were equal in all things except their p53 status, were not therefore compared. The time-point used for analysis was relatively early (48hrs) which may not have allowed for the differential effect of viral replication (maximal at 72 hrs) to take full effect [79]. The authors also acknowledge that their data on the mechanism of cell death, indicating a lack of apoptosis, was premature and incomplete. Even allowing for these criticisms, it is clear that further clarification of p53 requirements for cell death following dl1520 infection is required.

Other workers have also shown that the virus can replicate within some tumour cells of wild-type p53 status although in some cell lines there did seem to be a correlation with p53 status [127]. These workers further demonstrated that the virus could infect and replicate within some primary human cell lines including keratinocytes and fibroblasts. The levels of replication were relatively small and only noted at high multiplicities of infection (MOI). Again these results call into question many of the original assumptions concerning dl1520, although it has always been proposed that the virus could exist in p53 competent cells but in a way that would lead to cell death by apoptosis rather than viral replication leading to cytolysis.

One research group has suggested that the specificity of dl120 can vary according to the dose of the agent used. In experiments using two human hepatic carcinoma cell lines of p53 wild-type and mutant status it has been shown that dl1502 has a p53 selective effect on cytolysis of these cells in a tissue culture model. The selective effect is reduced at higher multiplicities of infection where the difference between the two cell lines is much less [128]. The same group further demonstrated that dl1502 could cause marked tumour regression in the p53 mut cell lines in a murine xenograft model but had no effect in the wild-type cell lines. These results would indicate that under the likely clinical conditions of use, where a relatively low titre of virus would be expected to reach each tumour cell, the effect of the virus would be p53 dependent. This p53 selectivity might be lost at higher MOI's although this was only demonstrated in an in-vitro tissue culture model.

The above contradictory results could be explained if it is considered that E1B-55K protein has several functions, some of which are p53 dependent and some of which are p53 independent [88]. These workers found that altering the temperature at which the virus was inoculated into cells could affect the p53 sensitivity of the virus' actions. They hypothesised that this indicates the E1B-55K protein has at least two separate functions at lower temperatures, one of which is independent of p53 function.

[129] provides a different explanation of the varying results described above [129]. This group studied the infectivity of the dl1520 and wild-type adenovirus in various cell lines of known but varying p53 status. They found that the cytopathic effect of the virus correlated with the infectivity of the virus in each cell line rather that the p53 status of cells. They also discovered that the expression of various early adenovirus proteins was restricted in tumour cell lines infected with dl1520. This affected the selectivity of the virus for various cell lines. They conclude that the infectivity and expression of early viral products is the most important determinant of the virus selectivity for lysing cells rather than p53 status. Mutations in other key cell cycle regulation proteins may also affect the ability of a cell to undergo apoptosis following infection with dl1520. It has been found for example in experiments using mesothelioma cell lines that the efficiency of dl1520 to kill cancer cells was higher in $p14^{ARF}$ null cell lines, and could be reduced by transfecting wild type $p14^{ARF}$ into these cell lines [130].

It is clear that the dl1520 adenovirus has undergone extensive testing in a laboratory setting. Some experiments have indicated that the virus has a selective action against cancer cells, which is dependent on these cells having a dysfunctional p53 protein. Some groups have called this explanation into question and have even suggested that p53 might be required to allow cell death following inoculation of the virus. It is clear that a full explanation of the mechanism of action of the virus will require further investigations. Despite these ongoing controversies involving mostly in-vitro tissue

culture work, trials involving the dl1520 have continued in human subjects. By and large these trials have given encouraging results as detailed below.

1.9.1 Previous in vitro studies using dl1520

The dl1520 adenovirus has been extensively studied in tissue culture and murine xenograft models. It has been shown that dl1520 can grow in cell lines which lack functional p53. The virus has been shown to replicate with efficiency comparable to wild-type virus in tumour cell lines lacking functional p53 but with reduced efficacy (≅ 1%) in cell lines with normal p53 function [1]. Tissue culture experiments demonstrated no detectable cytopathic effect on normal human fibroblasts or tumour cells with functional p53. Further studies indicated that if p53 function was restored to a previously p53 null cell line the activity of dl1520 was reduced [6]. The same workers further demonstrated that it was the lack of expression of E1B protein that led to this selectivity by introducing this protein in a p53 competent tumour cell line resistant to dl1520. This rendered this previously resistant cell line sensitive to cytolysis by the dl1520.

The virus was also shown to cause shrinkage of human tumour xenografts grown on nude mice in a p53 dependent manner with evidence of viral replication and spread within p53 deficient tumours. No evidence of adenovirus survival was noted in p53 competent tumour xenografts injected with dl1520 in the same experiment. Further work demonstrated that the virus could be delivered systemically to cause restriction in tumour growth in a p53 dependent manner and that chemotherapy can augment this restriction of tumour growth [1]. It has further been shown in a xenograft model using immunodeficient mice that intra venous administration of dl1520 can cause regression of tumours and that the extent of this effect is related to the amount of virus detected in tumours [131].

Additional evidence of the p53 selectivity of dl1520 was demonstrated in an experiment

analysing distribution of the virus under various conditions. Using a p53 deficient cell line, inoculation of 5% of cells prior to tumour implantation prevented tumour growth and inoculation of only 1% of cells restricted tumour growth. In contrast, infection of p53 competent cell lines prior to tumour cell implantation had no effect on tumour formation [132]. Other work using lung cancer cell lines in a tissue culture model also noted that p53 deficient cell lines were up to 10 times more sensitive that p53 competent cell lines to the effects of dl1520 [7]. Using dl1520 against human glioma cells in a tissue culture model again showed that the virus had almost a 10 times greater efficacy in p53 deficient tumour cells compared to lines with a functional p53 [133].

1.9.2 Combination effects with other treatment modalities

The same group also demonstrated that dl1520 can act in a synergistic fashion with chemotherapeutic agents in these cell lines and that this effect was greatest using p53 deficient cell lines. Chemotherapy has been shown to act synergistically with dl1520 in lung cancer cell lines in a p53 dependent manner [7]. It is postulated that the virus may act to induce increases chemosensitivity, either by inducing cells to enter the cell cycle or via a p-53 independent manner. It is also known that many standard chemotherapeutic agents work preferentially against tumours with a functional p53 [19, 20]. The combination of chemotherapy and dl1520 as an anti-cancer regimen is therefore attractive as they can act in a complementary fashion.

Radiotherapy can also augment the action of dl1520 in a murine xenograft model, again in a p53 dependent manner. This has been demonstrated using two tumour cell lines matched for p53 status where the virus had a greater cytolytic effect on the p53 mut cell line [134]. This study shows that radiotherapy does not hinder viral replication, probably because the viral genome is much smaller that the human genome and therefore less likely to sustain radiation induced damage. As radiation is most effective against cells containing functional p53, radiotherapy is able to complement the effects of the dl1520

virus as this acts against p53 mutant cells. The combination therapy could therefore target both p53 mutant and wild-type cells [135].

The above results suggest that the dl1520 virus has an anti-cancer cell action in many different tumour cell lines and that the level of cell and tumour destruction is related to the p53 status of the tumour cells concerned. Recent work has further demonstrated that dl1520 causes both cytolysis and apoptosis in tumour cell lines. Apoptosis is less marked in p53 deficient cell lines, as would be predicted by the mechanism of action of the virus [136]. This suggests that the higher replication and cytopathic effect of the virus in p53 deficient cells is due to the lack of induction of apoptosis following viral infection thereby allowing a longer period of viral replication prior to cell death by lysis. This cytolytic effect of dl1520 is therefore greater in p53 deficient cell lines than in p53 competent cell lines although cell death can occur in both.

1.9.3 Previous clinical studies

Dl1520 has been studied in several phase I and II clinical trials in a variety of tumour types. Most of the studies have involved the direct intra-tumoural administration of the virus but other methods have also been successfully utilised.

The first clinical trial of the virus was initiated in patients with recurrent squamous cell tumours of the head and neck which were considered refractory to conventional therapy. These tumours had all recurred following surgery and/ or radiotherapy. The virus has been delivered by repeated intra-tumoural injection into these recurrent, refractory tumours. A phase I dose escalation study utilizing a single dose of dl1520 delivered intra-tumourally in these patients was successfully completed [137]. This showed that of 22 patients who received a single intra tumoural dose of dl1520 repeated 4 weekly at doses of 10^7 to 10^{11} pfu there were no dose limiting tonicities. An objective tumour response was seen in 5 patients with tumour stabilisation in 8 patients and the response

duration was between 4 and 12 weeks. Evidence of viral replication was demonstrated in 4 of 22 post treatment biopsies (4 of nine taken from subjects who had received > 10^9 pfu of virus) by immunohistochemistry and in-situ hybridisation. Some patients exhibited grade1/11 febrile illness related to viral administration but this was self-limiting.

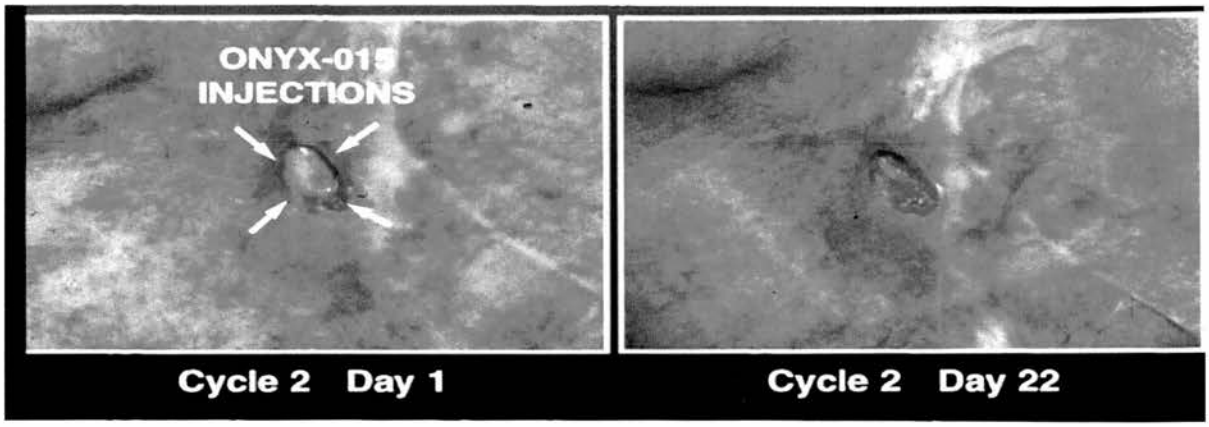
In a second phase I trial involving multiple intra tumoural injection a further 10 patients were treated with 5 receiving 5 daily injections of 10^9 pfu and 5 receiving 5 daily injections of 10^{10} pfu. No major differences in toxicity were noted from the dose escalation trial and there was one further objective tumour response. All of the tumours that responded had a mutant p53 sequence on gene sequencing. Evidence of viral replication was seen in 7 of 10 biopsies by immunohistochemistry and in-situ hybridisation. Patients tolerated the injection of virus well with no significant side effects. It is noteworthy that the injection protocol called for the virus to be injected into the healthy tissue surrounding tumour deposits as well as the tumour. No evidence of damage to this normal tissue was noted. In a phase II trial 30 patients went on to receive cycles of 5 daily injections of 10^{10} pfu of the virus with cycles repeated 3 weekly [138]. No additional toxicity was noted and there were 2 complete regressions and 2 partial responses in injected tumours. Symptomatic improvement was noted in 3 patients (improved jaw mobility n =2 and improved speech n =1).

Most recently a phase II study combining a similar regime of 5 daily injections of 10^{10} pfu with systemic chemotherapy (5-Fluorouracil plus cisplatin) has been completed [139]. Patients were suffering with recurrent, refractory squamous cell tumours of the head and neck that were otherwise considered untreatable. The trial protocol involved cycles of five daily injections of 10^{10} pfu of dl1520 directly into accessible tumour deposits. Patients received concurrent chemotherapy of intra-venous 5-fluorouracil plus cisplatin. Of 30 patients eligible for assessment 63% had a full or partial response to treatment. Patients with more than one lesion suitable for injection could have a saline

injection into a second lesion to act as a control. Of 11 subjects in this group 9 of 11 tumours injected with dl1520 showed a response compared to 3 of 11 saline injected tumours. This difference is significant indicating anti-tumour activity through the dl1520 and not simply due to the chemotherapy. No serious adverse reactions to the viral therapy were noted in any of these trials and results are encouraging that the virus has anti-tumour activity with no propensity to damage surrounding tissues. This trial again demonstrated that this therapy seems to be safe with an acceptable side effect profile. The level of clinical response was greater that would be predicted using chemotherapy alone suggesting that the virus could be used in conjunction with standard chemotherapeutic agents.

The virus has also been tested against various other tumour types. A trial looking at intra-hepatic arterial infusion of the virus in patients with liver metastases from gastro-intestinal tumours is currently under way [140]. No significant side effects thought to be due to the virus have been noted and in some cases there has been a reduction in the size of tumour mass in the liver. A clinical trial looking at direct injection of inoperable pancreatic tumours using ultrasound-guided needles is also currently ongoing [141]. A study using intra-venous dl1520 in patients with metastatic lung tumours is also underway [142]. These trials have produced promising data as to the feasibility and safety of this agent used with patients with advanced, solid malignancy.

Figure 1.3 – Example of the clinical effect of dl1520 in combination with chemotherapy. The picture shows a cutaneous deposit of squamous cell carcinoma from a head and neck primary cancer. Following injection with the dl1520 virus the tumour deposit has regressed.



1.10 Aims Of This Thesis

dl1520 is potentially a highly selective form of viral gene therapy that might act selectively against p53 deficient tumour cells. As more than 50% of human solid malignancies are deficient in p53 function this agent has potential as a novel, selective anti-cancer therapy [102]. This is a potential treatment for recurrent or systemic cancer as the virus is selective for cells with dysfunctional p53 and should not harm normal tissues. This selectivity is the key to the potential usefulness of the virus particularly if systemic delivery of the virus is to be possible. If the virus were to be used to treat disseminated disease then clearly some form of systemic delivery would be necessary.

1.11 Squamous cell carcinoma of the head and neck

Squamous carcinoma of the head and neck is a tumour type that is becoming increasingly common. It is associated with both smoking and high alcohol intake in the Western world and more commonly with the use of Betel nut in the Far East. Early cancers are amenable to primary treatment with surgery, radiotherapy or a combination of both. The disease however has a tendency to recur in the loco-regional lymph node basin, the cervical nodes. Once this happens the outlook for the patient is poor, with a five-year survival of only 30 %, which has remained unchanged for 30 years [143]. The disease seldom responds well to conventional chemotherapy agents and there is no effective treatment for recurrent disease [16].

Distal metastases are rare meaning that if the disease can be controlled locally this could lead to improved quality of life and possibly improved survival rates for patients. It is clear that a successful treatment for locally recurrent head and neck SCC is urgently required. Dl1520 could be well suited to treat disease in this area, as the cervical lymph nodes are amenable to direct intra-viral injection. As p53 mutations are very common in recurrent head and neck SCC this agent could be particularly suited to treating this

disease [144].

Although it is clear that dl1520 has an anti-cancer action there has been recent controversy as to the mechanism of action of the virus. One recent paper has presented findings suggesting that not only can dl1520 act to destroy cells with a functional p53 but that p53 may be necessary for virus mediated cell death in a tissue culture model [126]. Another group have suggested that the ability of the virus to cause cell death is independent of p53 status and that damage to normal cells is possible at high MOI's [127]. One group has suggested that the ability of the virus to infect and enter cells could be the key to its selectivity rather than the p53 status of a cell [129]. Other workers have demonstrated findings suggesting that the p53 selective effects of dl1520 disappear at high viral titres in a tissue culture model [128]. It has also been suggested that lack of function of the E1B protein could in itself inhibit viral production by restricting late viral mRNA production [88]. These findings contradict the results of many experiments with dl1520 but despite these controversies there is little doubt that the virus has a selective anti-tumour effect although the precise mechanism of this is not fully understood.

It is important to further investigate to what extent the virus can target tumour cells selectively. Without this selective effect, systemic use of this virus might lead to severe side effects if the virus can replicate within and destroy normal cells with a functional p53. If the agent were however truly p53 selective it would have great potential as an anti-cancer agent that is targeted specifically to tumour cells. Further clarification is desired as regards many of the above points. This is especially so as concerns the p53 and therefore tumour selectivity of the virus. The work reported in this thesis can be divided into the clinical trial undertaken and laboratory based experiments.

1.12.1 - Clinical trial

A clinical trial was devised to further investigate:

- **The mechanism of action of dl1520**
- **The p53 specificity of the virus in vivo**
- **Optimisation of intra-tumoural viral delivery**
- **Tolerance of administration of dl1520 in a select patient group**
- **Immune cell response to the virus**
- **Level of tumour necrosis following viral administration**

1.12.2 – Laboratory based experiments

Laboratory experiments were performed to further clarify:

- **The mechanism of action of dl1520**
- **The p53 specificity of the virus both in vitro and in vivo**
- **Optimisation of intra-tumoural virus delivery**
- **The effect of combining the virus with other standard chemotherapeutic agents**

There follows below a synopsis of the clinical trial and laboratory experiments to further investigate the specificity and activity of dl1520 particularly as this relates to p53 status of cells. A tissue culture model with cell lines matched for p53 status was used to study the effect of combining chemotherapy with dl1520 in-vitro. A murine xenograft model was designed to investigate the effect of intra-tumoural injection of dl1520 in different cell lines matched for p53 function.

1.13 Clinical trial

A clinical trial was devised to explore the p53 selectivity of dl1520 following a single intra-tumoural injection into oral cancers at set points prior to the tumour's resection.

Virus was injected into half of each tumour and saline into the other half to act as a control. An injection of virus into an area of normal tissue within the oral cavity was also made. This was made into an area of grossly normal tissue not affected by tumour or any other lesion. Following resection samples were analysed for viral replication and spread; oncolytic effect; mechanism of cell death and expression of p53 and p21 protein. Results were correlated with p53 status determined by gene sequencing of tumour samples taken prior to viral injection.

The effect of the injection of the virus on the patients' immune system was also investigated. FACS analysis of whole blood samples to detect various cell markers was undertaken before and after the viral injections. This allowed measurement of various immune cell sub-sets in the circulation to determine what effect the virus has on these following intra-tumoural injection.

1.13.1 Laboratory based experiments

1.13.2 Effect of combining dl1520 with chemotherapeutic agents

Many human malignancies are treated with systemic chemotherapy either as adjuvant treatment to a primary therapy or to treat recurrent or metastatic disease. We wished to ascertain the effect of using dl1520 as an anti-cancer agent along with chemotherapy. It is important to determine if the virus could act in synergy or in an additive fashion with standard chemotherapeutic agents or if there was any evidence of antagonism. A tissue culture model was devised using cell lines matched for p53 status. These were exposed to various concentrations of dl1520 for different time points to allow for any possible differential effect caused by replication over time. Cells were then treated with the chemotherapy agents at various concentrations. Clonogenic analysis was used to determine levels of cell kill at each level of viral load and concentration of chemotherapy. Results looking at the clonogenic survival of sub-cultures for each value

were determined against controls with no virus or chemotherapy agent.

1.13.3 Effect of a single intra-tumoural injection of dl1520 in a xenograft model

An experiment to investigate the effect of a single intra-tumoural in a murine xenograft model was devised. Cell lines matched for p53 status were used to grow xenografts to a size of approximately 6mm. These were injected with a single injection of dl1520 into all 4 quadrants of the tumour to investigate the effect of p53 status on viral distribution and replication. Tumours were harvested at various time-points following the injection to look at effects due to replication as well as distribution of the virus.

1.13.4 Effect of varying the carrier medium on distribution of dl1520 adenovirus on a single intra-tumoural injection of dl1520 in a xenograft model

Previous work has shown that following injection of dl1520, both in xenograft models and whilst treating human subjects, the virus tends to be restricted to an area between the necrotic centre of the tumour and the viable tumour tissue. This is thought to be the interface where the cytolytic effect of the virus is at its maximum. If the virus could be distributed more fully throughout the tumour it is possible that this would increase the biological effect of the virus as its activity would not be restricted to this area.

Several agents are in use clinically which could in theory increase the distribution of an intra-tumurally injected agent. Hyaluronidase is an enzyme that acts to break down hyaluronic acid, an important constituent of interstitial ground substance. This causes elements of the connective tissue to liquefy, aiding the distribution of any agent injected in conjunction with the hyaluronidase. Hyaluronidase is in use clinically to increase the spread of local anaesthetic agents injected subcutaneously and is known to be effective in this action [145]. Lignocaine is a commonly used local anaesthetic agent that acts by

blocking sodium channels. This also has the effect of dilating arterioles thus causing increased local blood flow by vasodilatation. Increased local blood flow could lead to enhanced distribution of an intra-tumurally injected agent such as dl1520.

An experiment was devised to measure the effect of varying the carrier medium on the distribution of dl1520 following a single intra-tumoural injection in the xenograft model. Hyaluronidase was used as one agent as its effect in breaking down ground substance may increase viral distribution. Lignocaine was also used as its vasodilatory effect may act to increase viral distribution. A single injection of dl1520 was administered into all four quadrants of tumour xenografts. Tumours were harvested at set points following this injection and imunohistochemical staining used to determine the distribution of virus at these time points.

CHAPTER 2

A CLINICAL TRIAL INVOLVING A SINGLE PRE-OPERATIVE INJECTION OF dl1520 IN RESECTABLE SQUAMOUS CELL MALIGNANCY OF THE HEAD AND NECK

A Clinical Trial Involving A Single Pre-operative Injection Of dl1520 In Resectable Oral Squamous Cell Malignancy

2.1.1 Background

There have been several clinical trials involving the administration of dl1520 to treat various cancer types including head and neck, ovarian and various gastro-intestinal malignancies [75, 146]. All such trials have involved patients with either locally advanced or metastatic disease not treatable by conventional means. Methods of virus administration have included direct intra-tumoural injection, intra-peritoneal infusion, intravenous infusion and infusion into the hepatic artery to treat hepatic metastases. Several of these studies have shown encouraging results with the treatment well tolerated and showing efficacy in many cases in terms of regression or stabilization of disease. Side effects have been minimal with none of the above trials seeing a dose limiting toxicity with the agent. Some patients have suffered symptoms such as fever, malaise, nausea and headache, which are associated with adenoviral infection, but overall the treatments have been well tolerated.

In the field of head and neck cancer both phase I and phase II trials involving the virus alone and a phase II trial involving the virus plus chemotherapy in patients with recurrent tumours considered untreatable by conventional methods have been successfully undertaken [137, 139]. When used as a single agent there was evidence of a p53 selectivity of action of dl1520 as tumours that responded to the treatment were found to have mutant p53. In the trial combining chemotherapy with intra-tumoural injection there was no such selectivity demonstrated. This could be predicted as the chemotherapy agents used, cisplatin and 5-fluoruracil, are most effective in p53 competent tissues. In tumours with wild-type p53 therefore one could surmise that the chemotherapy would be more effective than in p53 null tissues where the dl1520 would be most effective.

Our study aimed to address the following main points:

1. To determine if the virus is selective for survival and replication in p53 mutant tumours as opposed to p53 wild type tumours in the clinical setting
2. To determine the level of viral spread and replication within such tumours.
3. To determine the effect of the virus on normal tissues following a direct viral injection

To answer these questions the following points were addressed:

1. What is the frequency of mutation of the p53 gene in tumour and normal tissue?
2. How does the presence of virus vary according to the p53 status of both normal and tumour tissues and to what extent can the virus spread through such tissues?
3. What are the levels of virus induced apoptosis in normal and tumour tissues following virus injection?

2.1.2 Viral replication

Much is known about the capacity of dl1520 to replicate in cell lines and tumour xenografts from laboratory based studies, as detailed above. Difficulties have however been encountered in attempts to ascertain accurate data as to levels of viral replication and spread in various tissues and how this relates to tissue p53 status in the clinical setting. In the various clinical trials involving head and neck malignancy for example there were difficulties in obtaining representative tissue biopsies from the heterogeneous tumour samples. Tumours in this trial were recurrent and usually large making representative sampling difficult. The biopsies taken were small trucut biopsies that were not necessarily representative of the relatively large tumour masses being sampled.

Analysis of these samples from these trials has so far given little information as to p53 specificity of dl1520, levels of viral replication, spread within tumours and mechanism of cell death in the clinical model [131]. Biopsies have shown evidence of viral presence following treatment but data as to viral replication as opposed to viral presence is at

present limited.

2.1.3 Viral spread

Little is known about the propensity of the virus to spread within tumours following injection. In-vivo studies in a murine xenograft model have suggested that the virus can spread following injection. In clinical use there would be many other factors that would influence this such as the physical properties of the tumours and the host immune response. Any spread of the virus within tumour tissue could be beneficial in allowing widespread tumour cell kill of widespread disease even if this tissue was not directly injected. If the virus was capable of spreading within normal tissue however this could lead to collateral damage of healthy tissues surrounding the treated tumour so adversely affecting the side effect profile.

To date, studies of intra-tumoural injection of the virus in head and neck tumours have dealt with tumours that have been previously treated with radiotherapy, which usually leads to tissue fibrosis. In effect the tumours were surrounded by the physical constraints of fibrotic tissue. This may have had the effect of preventing optimal distribution of the virus. The tumours were generally large which meant assessment of spread was difficult, as only small trucut biopsies were available for analysis.

2.1.4 Effect of dl1520 on normal, non-malignant tissue

Little is known about the clinical effect of injecting dl1520 into normal healthy tissue. In theory any such injection would lead to only a very localized controlled apoptotic cell death without viral replication and spread as the action of the virus would be curtailed by the function of p53 in healthy tissue. Evidence of cytolysis of some wild-type cell lines has been noted in previous laboratory studies. Prior clinical trials of injection of dl1520 in recurrent head and neck tumour masses have involved an injection into tissues surrounding the tumour masses i.e. at the interface between tumour and healthy tissue

surrounding the tumour [139]. There was no clinical evidence of harm to these surrounding tissues in terms of ulceration or tissue destruction, which supports the hypothesis that the virus should do these tissues no harm. No biopsies of these normal tissues however have been analyzed for viral spread or p53 status.

We wished in this trial to determine the effect of a direct injection of dl1520 into normal tissues to see if the virus could harm normal tissue or if the effect would be contained. It is clearly important that further information as to the ability of the virus to replicate and spread within both malignant and normal tissue is obtained. Ideally these tumours would not have had previous radiotherapy which could limit the spread of the virus. This information needs to be correlated with p53 status of these various tissues.

2.1.5 Immune response

Prior studies have demonstrated a humoral response to adenoviral injection, which was present following intra-tumoural injection of the virus [137]. Administering a virus into a patient is likely to stimulate both a humoral and cell mediated immune response as the body recognises the virus as a pathogen, [147]. Such an immune response could limit the effectiveness of viral therapy particularly where multiple doses are administered, as circulating anti-bodies would quickly destroy the virus. A strong immune response against adenovirus could curtail efforts to deliver a viral therapy systemically.

Localised immune cell infiltration into tumour however could lead to enhanced tumour cell destruction. A gene therapy study looking at adenoviral injection into breast and melanoma cutaneous deposits has demonstrated cytolysis associated with immune cell infiltration, although it was unclear whether the adenovirus or the gene product subsequently produced was the cause of this [148]. We wished to investigate in more detail the immune response generated against the virus following a direct intra-tumoural injection by analysing white cell counts before and after injection of the virus.

2.1.6 Mechanism of cell death

It is postulated that the dl1520 virus should cause cell death predominantly by cytolysis following viral replication in tissues which are permissive for such replication i.e. p53 null cells. It is known that dl1520 can also cause apoptosis and this would be predicted in p53 competent tissue. It is therefore important to know to what extent the virus can generate apoptotic cell death in both healthy and cancerous tissue and how this relates to p53 status of these tissues.

2.2 Trial design

In the hope of answering the above questions a clinical trial was designed and implemented. The trial was devised to investigate a single intra-tumoural injection of dl1520 into tumour tissue and normal tissue in patients with squamous carcinoma of the oral cavity was undertaken. Patients due to have excisional surgery for intra oral squamous carcinoma were recruited into three cohorts who received a direct intra-tumoural injection of virus at either 1, 3, or 14 days prior to excision of the tumour. Patients also received an injection of dl1520 into normal intra-oral tissue at this time. The site of this normal tissue injection was buccal mucosa in an area of grossly normal tissue not involved by tumour or any apparent field change within the mouth. The Local Research Ethical Committee, the Gene Therapy Advisory Committee and the Medicines Control Agency approved the experimental protocol.

Surgery was undertaken as per the normal protocol for such cases, involving excision of the primary tumour and in most cases the cervical lymph nodes from the side of the neck closest to the tumour [149]. A biopsy of the area of normal tissue injected with the virus was taken at this time. Following surgery the tumour and the biopsy of the injected normal tissue were analyzed to look at viral replication and spread within the samples. The samples were also tested for p53 status; p21 status and the level and mechanism of cell death. Peripheral blood counts including lymphocyte counts were taken prior to

injection and immediately prior to surgery.

By analysing tumour samples at the three time points it was hoped to determine levels of replication of dl1520 virus by looking at the difference between levels of virus at 24hrs, 72hrs and 14 days. Earlier studies utilizing a mouse xenograft model have demonstrated that in this model the virus peaks at a maximal level at around 72 hrs and then levels would be expected to tail off by day fourteen. This would be predicted by the replication cycle of the adenovirus [79].

This study was designed to give important information about the ability of the virus to survive and replicate in both p53 deficient and p53 functional tumour tissue and normal, non-tumour tissue and allow us to correlate this with cell death of both tumour and normal tissue. It also allowed us to determine the nature of the body's immune response to viral injection both systemically and within the tumour itself.

2.3 Oral squamous cell carcinoma

All the patients recruited into the trial were suffering from oral squamous cell carcinoma. These tumours have frequent p53 mutations and are accessible for direct injection and as such were a suitable model for this study [90]. Oral SCC is a relatively uncommon tumour in the West but is increasing in incidence, especially amongst females and younger adults < 40 yrs [91, 150]. The disease can be aggressive and often recurs loco-regionally following primary treatment. Distal metastases are however rare and this tumour could be an ideal candidate for intra-tumoural therapies such as dl1520 as both the primary tumour and likely sites of recurrence are amenable to direct injection.

Oral SCC is thus an ideal candidate for investigation of dl1520 viral gene therapy. The tumour is amenable to injection and is suitable for the assessments required, as detailed above. There is a need for an effective salvage treatment for this form of cancer as if the

tumour recurs following primary treatment it is not usually amenable to further standard treatments [15, 151]. Targeted gene therapy strategies such as dl1520 can potentially address the problems posed by such tumours.

Figure 2.1 – a typical example of intra-oral SCC prior to injection with dl1520 adenovirus

CHAPTER 3

PRE-OPERATIVE INJECTION OF dl1520

ADENOVIRUS - RECRUITMENT AND EVENTS

3.1 Recruitment criteria

Patients were eligible to be enrolled into the trial if they satisfied the following criteria:

3.1.1 Tumour status

1.Tumours had to be primary squamous cell carcinomas of the head and neck. All patients recruited in fact had cancer of the oral cavity including oropharynx.
2. The tumour had to be amenable to complete surgical resection.
3. The tumour had to be appropriately treated by surgical resection (as determined by the consultant in charge of each case).

3.1.2 Patient characteristics

1. Patients had to be 18-75 years of age.
2.Consent for study participation was given before screening and treatment, on an approved informed consent form.
3. Hepatic AST and ALT < 2.5-fold upper limit of normal; total bilirubin within normal limits; PT/ INR = 2.0 and PTT within normal limits.
4. Karnofsky Performance Status ≥ 70%.
5. For sexually active males and females of reproductive age a suitable contraceptive had to be used during the conduct of the trial and for three months afterwards.

3.1.3 Exclusion criteria

Patients with any of the following were excluded from the study.
1. Ulceration and/or necrosis of the injected tumour might lead to difficulties in surgical resection, in the judgment of the P.I. and/or attending surgeon.
2. Invasion of the tumour into the carotid artery or its sheath.

3. Chemotherapy within the last three weeks (six weeks for nitrosoureas or mitomycin-C).

4. Radiotherapy to the target tumour site within the last three weeks.

5. Concomitant haematological malignancy (e.g. chronic lymphocytic leukaemia, non-Hodgkin's lymphoma).

6. Impending airway obstruction or other condition requiring urgent (predicted within two weeks) tumour debulking.

7. Pregnant or lactating females.

8. Maximal tumour diameter of 6cm.

9. Treatment with any other investigational therapy within the last six weeks.

10. Viral syndrome diagnosed within the last two weeks.

11. Ongoing active infection, including human immunodeficiency virus.

12. Patients with prior therapeutic adenovirus treatment.

3.2 Sample size

A sample size of 15 patients was recruited into the study. This was determined by the logistics of the supply of the dl1520 virus that was made available for this project by the sponsoring company. It was felt that this number would give sufficient numbers of the groups of patients required for analysis.

3.3 Screening investigations

A checklist of screening and pre-treatment evaluations follows:

1. Signed Local Research Ethics Committee approved informed consent
2. Testing of tumour biopsy by immunohistochemistry (IHC) and p53 gene sequencing for p53 status (results not required prior to patient's treatment with dl1520).
3. Complete medical history

4. Complete physical examination, including vital signs, weight, and height
5. Karnofsky performance score
6. Hematological tests, including prothrombin time (PT) and INR, and partial thromboplastin time (PTT) (Reference Section 10.1.1)
7. CD3, CD4, CD8 and total lymphocyte counts
8. Serum chemistry tests (Reference Section 10.1.2)
9. Plasma antibody to type 5 adenovirus (neutralizing)
10. Archival plasma sample
11. Electrocardiogram (12-lead)
12. Chest x-ray
13. Delayed-type hypersensitivity skin testing using a subcutaneous injection of Multitest CMI (testing response to: tuberculin, tetanus, diptheria, streptococcus, glycerin, candida, trichophyton and porteus antigens).
14. Secondary diagnoses
15. Baseline medical events, including signs, symptoms, and illnesses

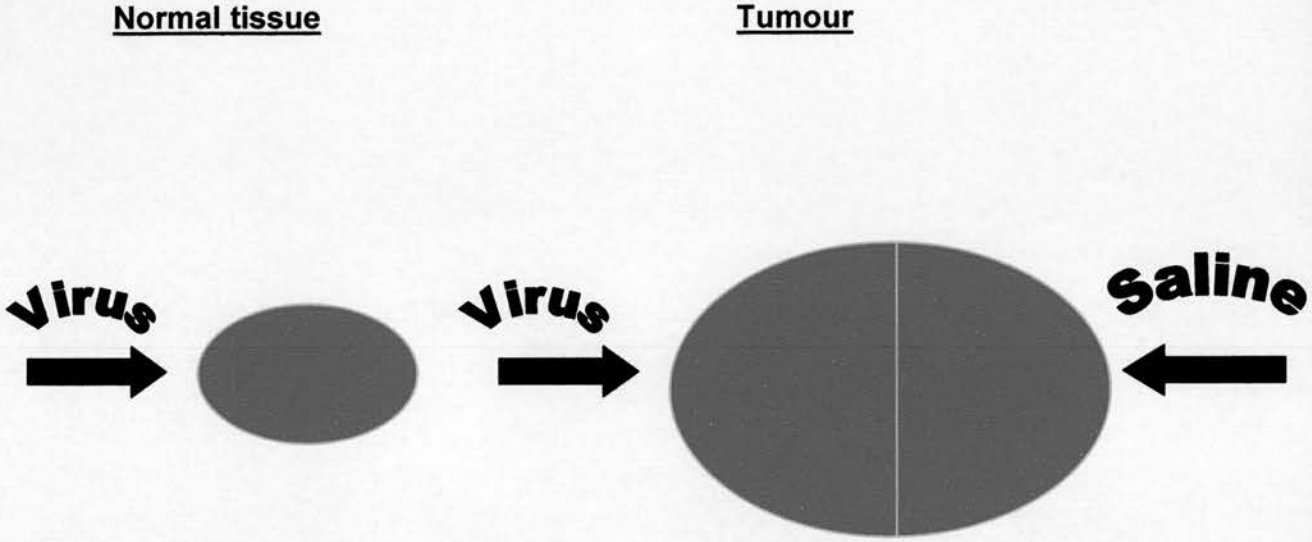
3.4 Treatment protocol

All patients were treated with 10^{10} pfu of virus into the tumour and a further 10^{10} pfu of virus into an area of normal intra-oral tissue, (buccal mucosa). The patients were enrolled into the study and then randomized to receive surgery for tumour excision and normal tissue biopsy at specified timepoints following viral injection. This involved three groups of five patients being allocated to receive an injection of dl1520 virus at fixed times prior to surgery as follows:

- 5 patients received a dl1520 injection 24 hours (+/- 6 hours) prior to surgery.
- 5 patients received a dl1520 injection 72 hours (+/- 24 hours) prior to surgery.
- 5 patients received a dl1520 injection 14 days (+/- 1 day) prior to surgery.

The injection protocol called for the tumour to be divided into halves. One half of the tumour was injected with virus and the other half (control half) was injected with diluent only. The volume of liquid injected into each hemi-tumour was calculated as 15% of the entire tumour volume. The volume injected into the normal tissue was 100µl in each case. Tattooing was used to mark the areas of each tumour injected and the area of normal tissue injected so as to ensure the correct areas were sampled in each case. 1% lignocaine could be administered in to the tumour and/ or normal tissue prior to the injection as a local anaesthetic (see figure 3-1).

Figure 3.1 – injection protocol



Following the injection patients were monitored for any adverse events and were kept in hospital for a minimum of 24 hrs. Both injection sites were inspected prior to discharge for evidence of inflammation or necrosis. Patients underwent surgical resection of their tumour at a given time-point following the injection of dl1520 (24hrs, 72hrs or 14 days). Immediately prior to surgery blood samples were drawn for CD3, CD4, CD8 and total lymphocyte counts. A biopsy of the area of normal tissue injected with dl1520 was also taken at this time. At the time of surgery each tumour specimen was divided into an Onyx injected half and a control or diluent injected half and from each a sample of tumour was taken for a frozen specimen. The remainder of each hemi-tumour was fixed in formalin as per the normal histology protocol.

Following surgery the patients received the normal standard of care following each operation. A note of adverse events was taken and patients were reviewed regularly following recovery.

CHAPTER 4 – MATERIALS AND METHODS

CLINICAL TRIAL

4 Materials and methods

4.1 Protocol for lymphocyte counts - whole blood

500 µl of whole blood was aliquoted into 5 ml analysis tubes. Anti-bodies were supplied by Becton-Dickinson. To each of 8 tubes 10 µl of antibodies were added as follows:

Tube 1 - both colour irrelevant i.e. PE/ FITC.
Tube 2 - CD3/ PE -ve.
Tube 3 - CD4/ FITC -ve.
Tube 4 - CD3/ CD4.
Tube 5 - CD2/ CD8.
Tube 6 - CD4/ CD14.
Tube 7 - CD3/ RO.
Tube 8 - CD3/CD56.

Tubes were incubated at 4 °C for 20 minutes. 2ml of FACS lysis solution (1:10 dilution dH2O) was added and samples left for 10 minutes at room temperature. Samples were then centrifuged at 1200rpm for 5 minutes, the supernatant aspirated and the pellet resuspended. 2ml PBS was added and the samples were re-centrifuged at 298 RCF for 5 minutes. The supernatant was again aspirated and the pellet resuspended in 500µl PBS. FACS (fluorescence-activated cell sorter) analysis was performed on each sample using a Becton Dickinson FACS cytometer.

4.2 Sample processing

4.2.1 P53 gene sequencing

Tumour biopsies for gene sequencing were immediately flash frozen in liquid nitrogen. Gene sequencing was performed at Virco Laboratories, Baltimore, USA using the

genechip micro array. Samples from the normal tissue biopsies were not assessed for gene sequencing as the samples taken were small and it was felt that there was not enough tissue to spare to allow for gene sequencing. Probes on the array are arranged in sets of five. Each probe in the set is perfectly complementary to the reference sequence except for a mismatch position, called the "substitution position". At the substitution position, each of the four possible nucleotides (A,C,G,T) and a single base deletion are represented in the probe set. Assay conditions optimise hybridisation of the fluorescently labelled DNA target to the probe that best matches its sequence. The array contains probes that can test the entire 1.2-kb coding sequence of the *TP53* gene, including a part of the intron/exon boundaries [152]. This hybrid yields higher fluorescence intensity relative to the other four target probe hybrids in the set. There are probe sets complementary to every base in the target gene, so each base along the gene is examined for the presence of mutant sequence. The assay has been shown to have a concordance of 92% when compared with traditional techniques, and could detect as little as 1% mutated DNA [153].

4.2.2 Normal tissue biopsy and excised tumours

At the time of surgery the excised tumours and the biopsy of normal tissue were divided into two halves to represent the dl1520 injected normal tissue, the dl1520 and saline injected hemi-tumours. The normal tissue sample was bisected and from the cut face of each half a slice of tumour was removed. Each of the three samples was placed in a cryomould half filled with OCT solution, which had been briefly immersed in liquid nitrogen. The sample was then placed in the cryomould, covered in OCT and then frozen in liquid nitrogen for 30 seconds. The remaining tissues were fixed in formalin and processed in the normal fashion for histological, immunohistochemical and in-situ hybridisation analysis.

4.3 Protocol for in-situ hybridisation for adeno-virus DNA [154]

Paraffin sections were dewaxed in three changes of xylene for 5 minutes each and rehydrated through a series of 100%, 90% and 70% ethanol and distilled water. The slides were then dried in an incubator at 37°C for 10 minutes. They were then immersed in protienase K buffer at 42°C for 2 minutes. Slides were fixed in 4%formaldehyde in PBS for 5 minutes and dehydrated through dH2O, 70% ethanol and 100% ethanol before being dried at 37°C for 10 minutes. Each run was repeated to confirm accuracy. Both positive and negative control slides were included, positive slides being provided by the test manufacturer and archival oral SCC specimens were used as negative controls.

40µl of biotinylated adenoviral DNA probe was applied to each section and a coverslip applied which was sealed with cow gum. Hybridisation was carried out overnight at 37°C. Each coverslip was removed in PBS and the slides washed for 5 minutes. 100µl of post-hybridisation reagent was applied to each section for 10 minutes at 37°C and slides were rinsed in two changes of PBS.

Anti-biotin/ alkaline phosphatase conjugate diluted 1:200 with PBS with 0.1% Tween 20 was applied to each slide and incubated for 2 hours at room temperature and slides were rinsed in PBS for 2 minutes. The slides were rinsed in colour development buffer twice for 5 minutes. NBT/BCIP was used for detection and sections were treated for 45 minutes at room temperature until colour developed. Slides were counter stained with nuclear fast red and rinsed in H20. Sections were dehydrated through 70%, 90% and 100% alcohol prior to being immersed in three changes of xylene. Sections were mounted using permount.

4.4 Protocol for hexon stain for adenoviral protein expression (Heise 1995, protocol obtained from Onyx Pharmaceuticals)

Paraffin sections were dewaxed in three changes of xylene for 5 minutes each and rehydrated through a series of 100%, 90% and 70% ethanol and distilled water. Immersing the slides in 3% hydrogen peroxide in methanol for 45 minutes quenched endogenous peroxidase activity and the slides were rinsed in two changes of PBS. All samples were analysed twice. dl1520 injected murine xenograft samples harvested from prior experiments were used as a positive control and archival oral SCC samples as a negative control.

The sections were then treated with protease (1.7mg/ ml in PBS) for 20 minutes in a humidified chamber at 35 ° C and the slides were rinsed in two changes of PBS. Casein solution was used to block non-specific binding (diluted 1:10 in PBS). The primary anti-body mouse anti-adenovirus monoclonal antibody Ig1k isotype (Chemicon) was initially used at a dilution of 1:1000 and 100 µl was added to the section and a coverslip added. This was incubated for 45 mins in a humidified chamber at 35 ° C and slides were rinsed in two changes of PBS. For the tumour biopsies this was found to give insufficient sensitivity and the concentration of anti-body was increased to 1:500.

Subsequent antibodies were provided from the Biogenex Super Sensitive Immunodetection System, Biogenex. 100 µl of a biotinylated secondary antibody was added to each section and incubated for 20 mins at room temperature and slides were rinsed in two changes of PBS. Streptavidin-horseradish peroxidase conjugate (100 µl) was added to each section and incubated for 20 mins at room temperature and slides were rinsed in two changes of PBS. DAB was used as a chromagen with 100 µl added to each section for 8 mins. Slides were then rinsed in distilled water, counter-stained in Mayer's hematoxylin for 2 mins and dipped in Ammonia water for 10 seconds. Sections

were dehydrated through 70%, 90% and 100% alcohol prior to being immersed in three changes of xylene. Sections were mounted using permount.

4.5 Protocol for Tunel in-situ cell death detection [155]

Paraffin sections were dewaxed in three changes of xylene for 5 minutes each and rehydrated through a series of 100%, 90% and 70% ethanol and distilled water. 50 µl of proteinase K was applied to each section, which was covered with a coverslip, and incubated for 20 mins at 37 ° C. Slides were rinsed in two changes of PBS. Endogenous peroxidase activity was blocked using 0.3% H2O2 in methanol and cells were initially permeabilised using 0.1% Triton X-100 in 0.1% Sodium citrate at 4°C for 2 mins. This was found to lead to insufficient sensitivity and the step was modified to have 5 mins permiabilisation at room temperature. All samples were analysed twice with irradiated mouse tails used as positive controls and normal skin as negative controls.

Slides were rinsed in two changes of PBS and 50 µl of Tunel reagent was added and a coverslip applied. Slides were incubated in a humidified chamber for 60 mins at 37 ° C. 50 µl of converter POD was applied to each section coverslip applied. DAB was used as a chromagen with 100 µl added to each section for 10 mins. Slides were then rinsed in distilled water, counter-stained in Mayer's hematoxylin for 2 mins and dipped in Ammonia water for 10 seconds.

4.6 Protocol for immunohistochemistry stain for P21/ P53 [156]

Paraffin sections were dewaxed in three changes of xylene for 5 minutes each and rehydrated through a series of 100%, 90% and 70% ethanol and tap water for 2 minutes each. Endogenous peroxidase activity was quenched by immersing the slides in 0.1 % hydrogen peroxide in distilled water for 30 minutes and the slides were rinsed in two changes of PBS for 5 mins each. Antigen retrieval was performed by treating the slides

in a 650W microwave oven for 15 minutes whilst immersed in sodium tricitrate.

Vectastain ABC kits were used to provide link antibodies. Each section was covered with 100 µl of blocking serum and was incubated in a humidified chamber for 20 mins at RT.
This was tapped off each section in turn and 100 µl of primary antibody added as follows:

For p53 stain the antibody was used at a dilution of **1/1000 in PBS for 45 minutes at RT.**
For p21 stain the antibody was used at a dilution of **1/100 in PBS for 1 hour at RT.**

Sections were rinsed in two changes of PBS and incubated with 100 µl of biotinylated antibody in a humidified chamber for 30 mins at RT. They were then rinsed in two changes of PBS. 100 µl of ABC solution was added to each section and these were incubated in a humidified chamber for 30 mins at RT and then rinsed in two changes of PBS. DAB was used as a chromagen with 100 µl added to each section for 8 mins. Sections were rinsed in PBS and each section was covered with 100 µl of blocking serum and was incubated in a humidified chamber for 20 mins at RT. Antibody was applied as described above. Sections were rinsed in two changes of PBS and incubated with 100 µl of biotinylated antibody in a humidified chamber for 30 mins at RT, before being rinsed in two changes of PBS. 100 µl of ABC solution was added to each section and these were incubated in a humidified chamber for 30 mins.

Slides were then rinsed in distilled water. For p53 and p21 they were counter-stained in Mayer's hematoxylin for 2 mins and dipped in Ammonia water for 10 seconds. Sections were dehydrated through 70%, 90% and 100% alcohol prior to being immersed in three changes of xylene. Sections were mounted using permount. All analyses were performed twice. Irradiated mouse tail was used as a positive control and normal skin as a negative.

CHAPTER FIVE

CLINICAL TRIAL – RESULTS

5. 1 General

5.1.1 Administration of the virus

All patients tolerated the injection of virus well. In 4 patients the injections were made whilst the patient was under general anaesthetic. In the remaining 11 patients the injections were made whilst the patient was conscious. Local anaesthetic was used in 9 out of 11 of these patients. Patients experienced mild to moderate discomfort but in no cases did this interfere with administration of the virus.

5.1.2 Adverse events

Adverse events are summarised in table 5.1 below. No serious adverse events thought to be due to the virus were noted. One patient developed painless swelling at the injection site 3 days following the injection. Anti-biotics were administered for a suspected bacterial infection and the swelling settled. No evidence of viraemia such as pyrexia or rigours was noted and there were no allergic reactions in any patient. Other complications were noted in this series including one loss of free tissue transfer, 4 cases of bronchopneumonia, one fistula formation and one case of electrolyte imbalance. None of these were thought to be related to the administration of the dl1520 adenovirus. These complications were in keeping with post-operative complications following this type of major surgery.

Table 5.1 – adverse events

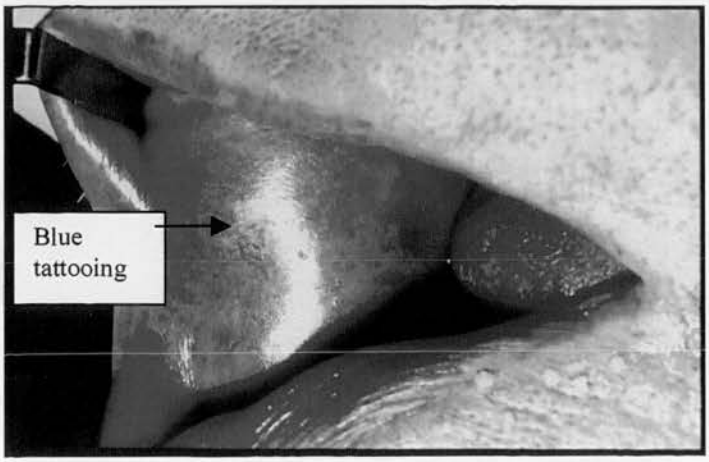
Nature of adverse event	Frequency	Relationship to virus
Swelling at injection site	**1**	**possible**
Flap loss	**1**	**unlikely**
Fistula formation	**1**	**unlikely**
Electrolyte imbalance	**1**	**unlikely**
Bronchopneumonia	**4**	**unlikely**

5.1.3 Clinical effect – tumour

The injected tumour samples were inspected regularly following injection and at the time of resection for any alteration in gross appearance. No gross changes were noted in tumour specimens following viral or saline injection. Tumours were assessed for decrease in size, inflammation or increased ulceration following the viral injection but there were no changes visible to the naked eye in either the dl1520 injected or saline injected hemi-tumours.

Figure 5.1 – resected tumour sample following injection of dl1520 72hrs previously

Figure 5.2 – buccal mucosa i.e. normal tissue following injection of dl1520 72hrs previously



5.1.4 Clinical effect – normal tissue

No permanent gross changes were noted in normal tissue specimens following viral injection. The area of buccal mucosa was assessed for signs of inflammation or ulceration following injection. In one case there was a transient inflammation of the injection site 72hrs following injection but no other changes visible to the naked eye in the dl1520 injected areas were noted. Figure 5.2 illustrates a typical injection site tattooed with blue dye for accurate biopsy of the injected area.

5.2 P53 sequencing of tumours

Of 15 biopsies taken for p53 sequencing, 14 were acceptable for gene sequencing. One sample was too small for accurate sequencing and was therefore unsuitable. The results are as noted below showing that 4 out of 15 biopsies showed a wild-type gene sequence with 10 showing one of a variety of mutations (see table 5.2). Also shown is the score for each sample for immunohistochemistry for p53 protein (see table 5.3). Mutations were cross referenced with a p53 mutation database, the International Agency For Cancer Research, to determine if the mutations had been described previously and if so what their likely effect was on the production or function of the p53 protein. Where known the site of the codon affected by the mutation was also described by cross-referencing with recent work looking at the structural significance of p53 DNA mutations on the eventual p53 protein formation [157]. All the mutations found had been previously noted in other solid tumours. All the mutations described were sufficiently important to disrupt the function of the p53 protein.

Table 5.2 – p53 gene sequencing

Patient Number	P53 sequencing	Effect on translation	Area of DNA affected	Likely effect
1	Wild-type			
2	Mutation missense exon 6 644G>T	Codon 215 from AGT (serine) to ATT (isoleucine)	Core domain	Likely effect on interactions with other proteins
3	Mutation missense exon 7 746T>G	Codon 255 from ATC (isoleucine) to AGC (serine)	Core domain	Likely effect on interactions with other proteins
4	Mutation missense Exon 5 488A>G	Codon 163 from TAC (tyrosine) to TGC (cysteine)	Core domain	Likely effect on interactions with other proteins
5	Inadequate sample			
6	Wild-type			
7	Mutation Intron 6 IVS6+2T>G	Modifies the consensus sequence of the splice donor site	Splice donor site	Alters pattern of MRNA splicing
8	Mutation missense exon 5 476C>T and mutation nonsense exon 10 1024C>T	Codon 159 from GCC (alanine) to GTC (valine) and codon 342 from CGA (arginine) to TGA (stop)	DNA binding domain β-strand	Affects DNA binding Truncation of protein

Table 5.2 (continued) – p53 gene sequencing

Patient Number	P53 sequencing	Effect on translation	Area of DNA affected	Likely effect
9	Mutation missense Exon 7 742 C>T	Codon 248 from CGG (arginine) to TGG (Tryptophan)	DNA binding domain (turn)	Affects DNA binding
10	Mutation Intron 5 IVS5-1G>C	Modifies the consensus sequence of the splice acceptor site	Splice acceptor site	Alters pattern of MRNA splicing
11	Wild-type			
12	Mutation Intron 8 IVS8-2A>G	Modifies the consensus sequence of the splice acceptor site	Splice acceptor site	Alters pattern of MRNA splicing
13	Mutation missense Exon 5 524G>A	Codon 175 from CGC (arginie) to CAC (Histidine)	DNA binding domain	Affects DNA binding
14	Wild-type			
15	Mutation missense Exon 5 536A>T	Codon 179 from CAT (histidine) to CTT (Leucine)	DNA binding domain (α-helix)	Affects DNA binding

All the mutations found had been previously described in human malignancy. They were all described as being likely to adversely affect the function of the p53 protein. The commonest mutations found involved missense mutations (n=7). Four of these were in the DNA binding domain, 2 were in the core domain and one was at codon 225. There were three mutations affecting introns 5,6 and 8 so as to alter the pattern of mRNA splicing which would have a significant effect on p53 formation. It is therefore likely that in all cases where a p53 mutation was noted in the tumour biopsy then the function of the p53 protein produced would be defective[158-161].

Table 5.3 - shows the correlation between the gene sequencing results from tumour biopsy and the expression of p53 found with immunohistochemical staining. The immunohistochemistry scores were derived from assessing a combination of the sum of % of cells positive within the sample and the intensity of the staining as follows:

% cells staining positive:	**Nil**	**1-20%**	**20-80%**	**>80%**
Score	**0**	**1**	**2**	**3**
Intensity of cell staining:	**Nil**	**Weak**	**Moderate**	**Strong**
	0	**1**	**2**	**3**

For each specimen therefore there is a minimum score of 0 and a maximum of 6. A minimum of five high powered fields (hpf) for each specimen were assessed. The samples were controlled for cell density as assessments were made of tumour tissue only which would be expected to have a similar density of cells. As the assessment was made on a percentage of cells being positive, the absolute cell density was not crucial to this score.

It can be seen that all the samples with a wild-type gene sequence were negative for p53 expression with immunohistochemistry. This would be expected as negative staining for p53 generally indicates a normal p53 protein, although excessive amounts of normal

protein can be detected by immunohistochemistry [162, 163]. Of the tumour samples where gene sequencing indicated a p53 mutation, 8 out of 10 stained positive with immunohistochemistry. There are several explanations for this discrepancy that are discussed in the next chapter.

Of the normal tissue biopsies 8 out of 15 stained positive for p53 mutation. This could indicate a possible field change in these areas, as it is known that p53 overexpression is common in areas of normal mucosa adjacent to oral cancers [4]. This might indicate that these areas harboured cells with the potential to undergo malignant change, although the significance of p53 overexpression in normal mucosa is not fully understood [3].

Table 5.3 Correlation of p53 immunohistochemistry with gene sequencing for p53

Patient Number	P53 sequencing	p53 tumour dl1520	p53 tumour control	P53 normal tissue
1	Wild-type	0	0	2
2	Mutation missense exon 6 644G>T	5	0	2
3	Mutation missense exon 7 746T>G	4	4	0
4	Mutation missense Exon 5 488A>G	0	3	0
5	Inadequate sample	2	3	3
6	Wild-type	0	0	0
7	Mutation Intron 6 IVS6+2T>G	3	3	0
8	Mutation missense exon 5 476C>T and mutation nonsense exon 10 1024C>T	4	5	0

Table 5.3 (continued). Correlation of p53 immunohistochemistry with gene sequencing for p53

Patient Number	P53 sequencing	P53 tumour dl1520	P53 tumour control	P53 normal tissue
9	Mutation missense Exon 7 742 C>T	6	6	4
10	Mutation Intron 5 IVS5-1G>C	0	0	2
11	Wild-type	0	0	0
12	Mutation Intron 8 IVS8-2A>G	0	0	0
13	Mutation missense Exon 5 524G>A	5	5	3
14	Wild-type	0	0	3
15	Mutation missense Exon 5 536A>T	5	4	2

5.3 Microscopic appearance of tissue

5.3.1 Normal tissue

The 15 normal tissue biopsies were similar in appearance viewed microscopically. There was no evidence of tissue necrosis or ulceration in the areas sampled. A lymphocytic infiltrate was noted in all the samples both within the epithelial layer and deeper into the muscle (see figure 5.5). This infiltrate was a constant finding at all three timepoints assayed and no difference was found between viral injected and control samples. It is known that oral SCC is often associated with a lymphocytic infiltrate and it is not clear if treatment with the dl1520 adenovirus has any effect on this [164].

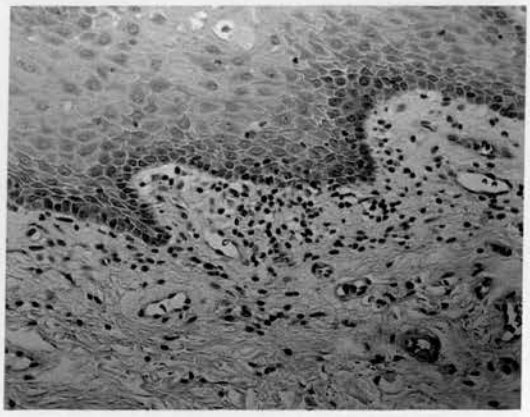
5.3.2 Tumour tissue – dl1520 injected

The tumour samples were heterogeneous in appearance with samples displaying a spectrum of tumour patterns and differing amounts of other tissues such as muscle and salivary glands. A marked lymphocytic infiltrate was noted in all specimens at all timepoints assayed as noted above. There was no evidence of grossly increased tumour necrosis in the dl1520-injected specimens compared to the saline injected areas.

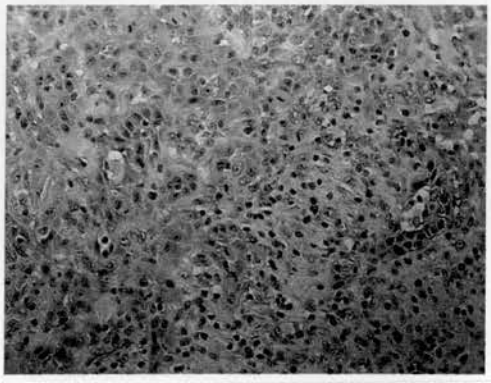
5.3.3 Tumour tissue – saline injected

The appearance of these samples was similar to that of the dl1520 injected samples. Again there was an obvious lymphocytic infiltrate but no evidence of gross tissue necrosis.

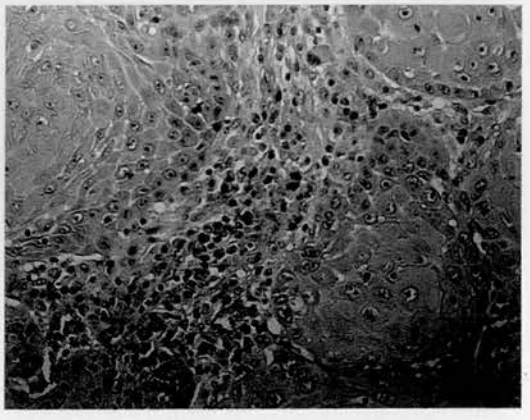
Figure 5.3 – H and E of normal tissue biopsy showing lymphocytic infiltrate



H and E of dl1520 injected tumour tissue sample showing lymphocytic infiltrate.



H and E of saline injected tumour tissue sample showing lymphocytic infiltrate



5.4 Adenoviral replication and distribution - detection of dl1520

Samples were assessed for the presence or absence of the dl1520 adenovirus as described above using both in-situ hybridisation (ISH) and immunohistochemistry (IHC). They were further analysed to determine the density of cells positive for viral detection per high-powered field in areas where virus was detected. All samples were assessed over the extent of the tumour cells. These areas were not formally controlled for cell density but as the assessments were of tumour cells only it can be assumed the cell density would be broadly similar. This is expressed as the average number of positive cells per high-powered field (range 0-63) +/- standard error. These results are summarised in figures 5.7 and 5.8. Both Student's t-test and Wilcoxan's rank sum tests were used to compare the results between the wild-type and mutant p53 tumour samples.

5.4.1 In-situ hybridisation (ish) for detection of adenovirus in tumour samples

ISH detected adenoviral DNA in 5 out of 10 samples with a mutation of p53 (50%) and 1 out of 4 with a wild-type p53 sequence (25%). The average density of virus found was 12.6+/-6 and 15.5+/-13 cells per high-powered field for mutant and wild-type tumour respectively (figures quoted +/- SEM). Virus was detected in 3 samples at 24hrs; 2 samples at 72hrs and 1 sample at 14 days. The average density of virus was at 16 cells per hpf at 24hrs; 9.2 at 72hrs and 12.4 at 14 days.

5.4.2 Immunohistochemistry for detection of adenovirus in tumour samples

Immunohistochemistry detected adenoviral hexon protein in 7 out of 10 samples with a

mutation of p53 (70%) and 1 out of 4 with a normal p53 sequence (25%). The average density of virus found was 9.9+/-3.7 and 7.5+/-6 cells per hpf for mutant and wild-type tumour respectively. Virus was detected in 4 samples at 24hrs; in 3 at 72hrs and in 1 at 14 days. The average density of virus was 9.6 at 24hrs; 10.2 at 72hrs and 6 at 14 days.

Virus was detected more often in p53 mut tumour samples using both ISH and IHC. A similar density of virus in each group was found using both techniques, the result being difficult to interpret as only one sample with a wild-type p53 sequence had virus detected, but this had a high density of cells positive. The difference in both detection rate and density between the mutant and wild-type samples is insufficient to reach statistical significance using either Student's t-test or the Wilcoxan's rank sum tests. Looking at the different timepoints it does appear that there is a trend for less virus to be found at day 14 compared to the earlier timepoints, but this difference is not statistically significant.

5.4.3 Detection of adeno-virus in saline injected tumour samples

ISH detected adenoviral DNA was detected in 2 out of 15 samples of saline injected tumour (13.3%) and immunohistochemistry detected adenoviral hexon protein in 3 out of 15 samples of saline injected tumour (20%). All of these had a mutation of p53. This shows that less virus is detected in saline injected samples as compared to virus injected tumours and this difference is statistically significant using the t-test and the Wilcoxan's rank sum test ($p=0.039$ and $p<0.01$ respectively). There is some evidence of spread of the virus from injected to non-injected areas but clearly this phenomenon is limited.

5.4.4 Detection of adenovirus in normal tissue samples

In the normal tissue biopsies ISH detected adenoviral DNA was detected in 2 out of 15

samples (13.3%). The density of cells positive for viral DNA was 12.5+/-5.5 for the tumour samples and 1.4+/-0.9 for the normal tissue samples. Statistical analysis using the t-test and the Wilcoxan rank sum test showed this to be a significant difference ($p=0.03$ and $p<0.02$ respectively). This demonstrates a significantly increased level of viral survival and replication in tumour samples than in normal tissue when assessed by ISH when looking at both frequency of any virus being detected and the density of virus in areas where it was present.

Immunohistochemistry detected adenoviral hexon protein in 2 out of 15 samples (13.3%). The density of cells positive for adenoviral hexon protein was 8.6+/-2.9 for the tumour samples and 0.6+/-0.4 for the normal tissue samples. Statistical analysis using the t-test and the Wilcoxan rank sum test showed this to be a significant difference ($p=0.009$ and $p<0.02$ respectively). This demonstrates a significantly increased level of viral survival and replication in tumour samples than in normal tissue as demonstrated by presence of adenoviral hexon protein. Gene sequencing was not performed on the normal tissue sample due to the relatively small volumes of tissue available, but immunohistochemistry for p53 status on all the samples staining positive for viral detection indicated the presence of abnormal p53 (see below).

Table 5.4 - Overall results for ISH to detect adenoviral DNA – wild-type v's mut p53

Sample type	No	Tumour dl1520 injected – density +/- SEM	Tumour saline injected density +/- SEM
Mut p53	10	12.6+/-6	1.3+/-0.4
Wild-type p53	4	15.5+/-13	0

Table 5.5 - Overall results for immunohistochemistry to detect adenoviral hexon – wild-type v.'s mut p53

Sample type	No	Tumour dl1520 injected - density +/- SEM	Tumour saline injected density +/- SEM
Mut p53	10	9.9+/-3.7	1.3+/-0.4
Wild-type p53	4	7.5+/-6	0

Table 5.6 - Overall results for dl1520 tumour v's normal tissue

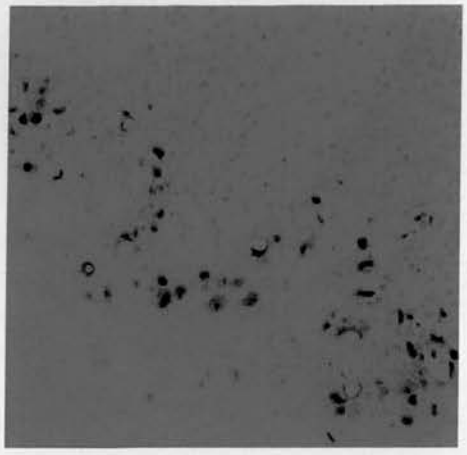
	Normal tissue viral density +/- SEM	Tumour tissue viral density +/- SEM
ISH	0.6+/-0.4	12.5+/-5.5
IHC	1.4+/-0.9	8.6+/-2.9

5.4.5 Overall viral detection

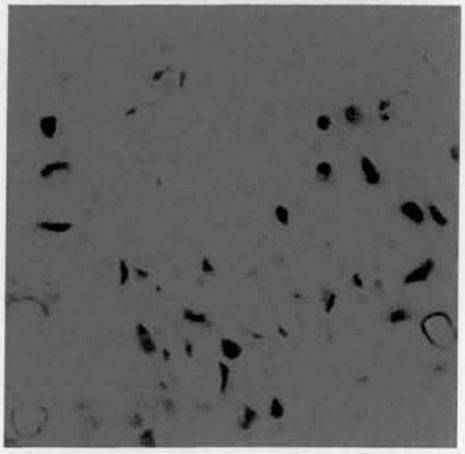
Combining the results of the ISH and IHC for hexon protein for the tumour samples it was possible to detect the virus in 10 out of 15 cases altogether (67%). This includes 8 out of 10 positive with mutant p53 (80%) and 2 out of 4 with wild type p53 (50%) (one sample was inadequate for gene sequencing). This compares to detection in 3 out of 15 normal tissue biopsies (20%). As described above the difference in detection between normal tissue and tumour tissue is statistically significant when either ISH or IHC are used. This clearly shows that the virus can exist preferentially in tumour tissue as opposed to normal tissue from a similar tissue type i.e. oral mucosa.

Figure 5.4 – examples of ISH for adenoviral DNA in a tumour sample injected with dl1520 72 hours prior to resection (p53 mutant sample)

X10 magnification



X20 magnification



X40 magnification (note evidence of cytopathic effect)

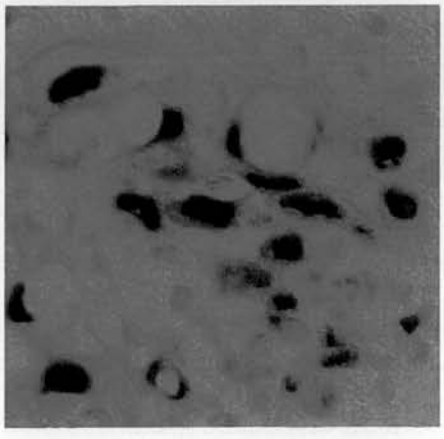
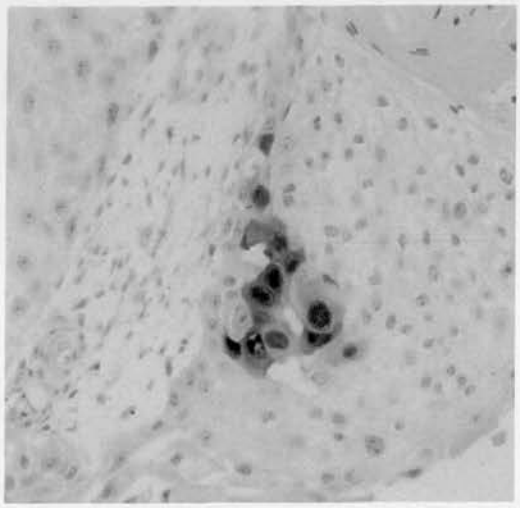
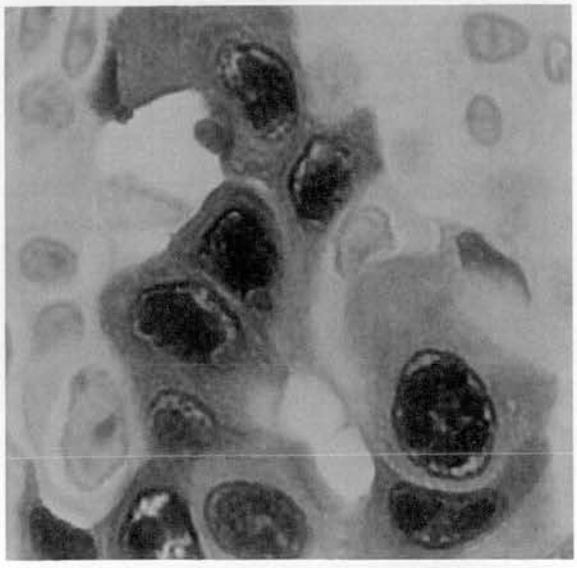


Figure 5.5 – examples of immunohistochemistry for adenoviral hexon protein in a tumour sample injected with dl1520 72 hours prior to resection (p53 mutant sample)

X10 magnification



x40 magnification – note evidence of cytopathic effect



5.5 Apoptosis levels

Levels of apoptosis in the dl1520-injected tumour samples were expressed as an average of apoptotic cells per high-powered field +/- SEM. A base line assessment of apoptosis was not obtained as consent for this additional biopsy was not covered within the ethical approval obtained for the protocol. In the tumours, fields were selected at the interface between invasive tumour and normal tissue. Malignant cells only were used for these assessments within the tumour samples. A minimum of five fields was assessed per tumour sample. In the normal biopsies apoptosis was assessed in the same manner with fields chosen between the epidermis and deeper layers. The values were as follows:

Table 5.7 – Tunel stain to detect apoptosis

	Tumour – dl1520 injected +/- SEM	Tumour – saline injected +/- SEM	Normal +/- SEM
24hrs	4.6+/-1.1	1.8+/-0.2	6.1+/-2.2
72hrs	1.7+/-0.52	2.5+/-0.5	5.3+/-3
14 days	2.8+/-1.2	2.5+/-0.5	3.2+/-1.6
All	3+/-1.1	2.3+/-0.42	4.9+/-1.3

Figure 5.6– apoptosis in a normal tissue biopsy

(X20 magnification) **(X40 magnification)**

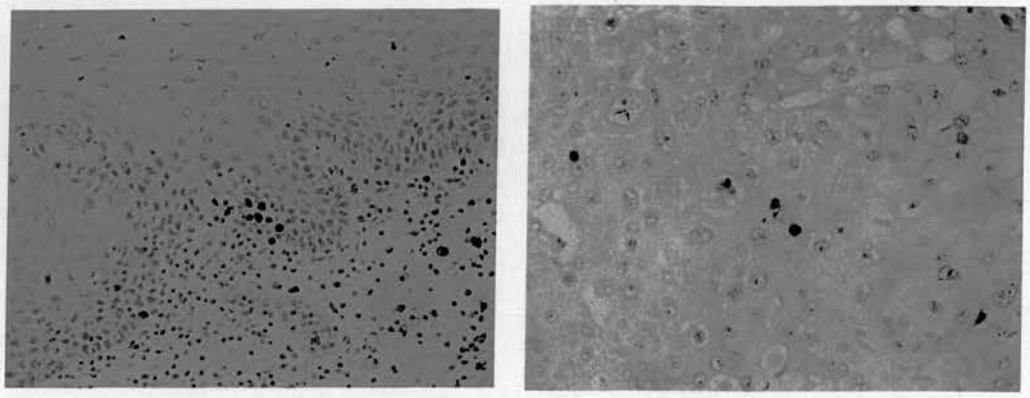


Figure 5.7 – apoptosis in a dl1520 injected tumour tissue biopsy.

(X20 magnification)

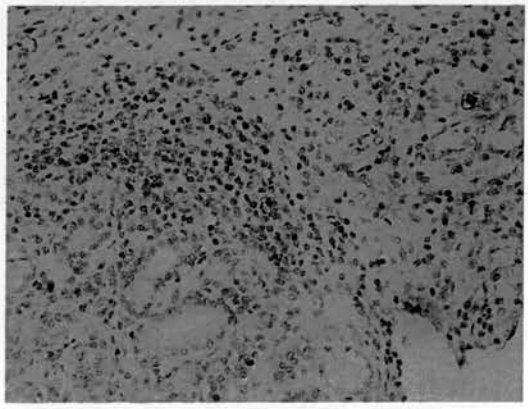
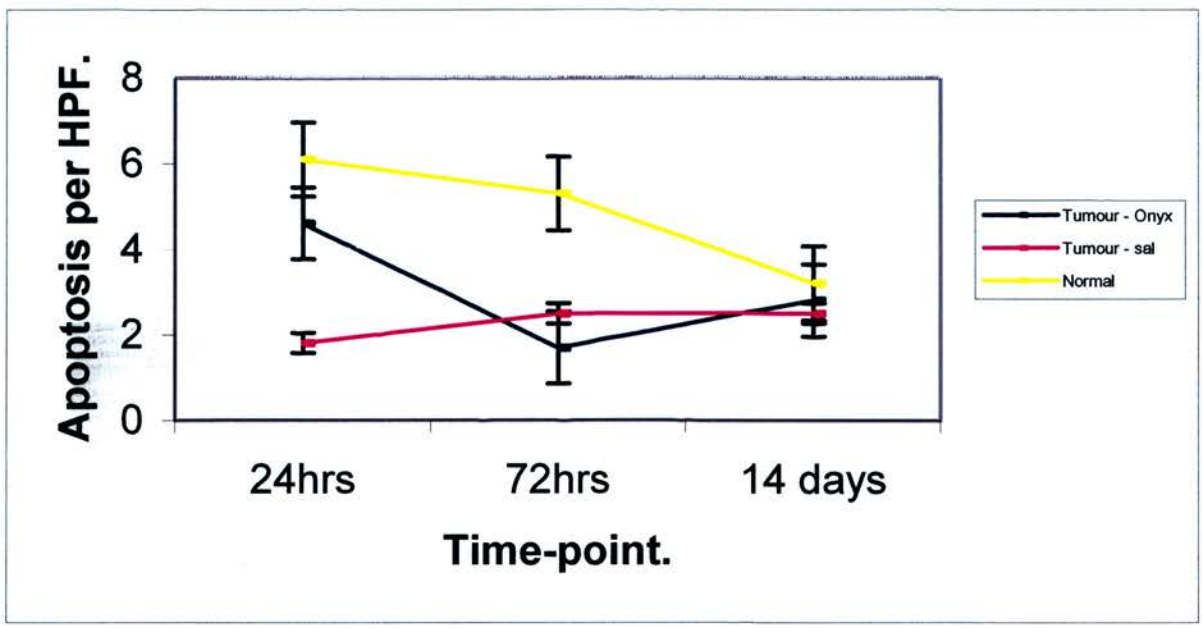


Figure 5.8 - showing levels of apoptosis in the different tissue samples at the time points indicated



The graph demonstrates an increase in apoptosis in the normal, dl1520 injected tissues as compared to the dl1520 or saline injected tumours at the 72 hr time-point. Comparing the values for dl1520 injected tumours and the normal tissue at 24 hrs and 72 hrs this difference is significant ($p<0.01$) (Wilcoxan's Rank sum test). This increased apoptosis is lost by the 14-day time-point where there is no significant difference between the normal tissues and the tumour tissues. There was no significant difference in apoptosis between the dl1520 injected and saline injected tumours. This suggests that the dl1520 virus can lead to high levels of apoptosis in normal tissue.

This might be expected as when adenovirus inoculates into a normal cell, i.e. that with a functional p53, the viral E1A protein activates p53 via a pathway involving the retinoblastoma protein [9]. This would therefore be expected to trigger apoptosis in such

cells as per the purported mechanism of action of the virus. Injecting dl1520 into normal tissue would therefore trigger an initial rise in cell apoptosis via this p53 pathway. This would not lead to viral replication in such tissues as the apoptosis triggered in this way prevents the virus replicating within these normal cells as the apoptosis effectively kills the host cell before replication can take place [80, 134]. Levels of apoptosis were not significantly different between viral and saline injected tumours. As most of the tumours had a defective p53 function this would be expected, as the virus would not trigger high levels of apoptosis in p53 deficient tissue.

When we look at tumour samples with wild type as opposed to mut p53 we find the following results:

Table 5.8 - Tunel stain for apoptosis by p53 sequence

Tumour type	Tunel +/- SEM
Mutant (n=10)	3.42+/-2.84
Wild-type (n=4)	2.55+/-1.12

The level of apoptosis in the mut p53 samples is 3.42 as compared to 2.55 for the wild-type samples. When either the t-test or Wilcoxan rank sum tests are applied this difference is not significant.

5.6 - P53 expression – immunohistochemistry

The results of immunohistochemistry were determined using the scoring system based on both the % of cells staining positive for p53 and the intensity of this staining as previously described. This scoring system gives a maximum score of 6 and a minimum score of 0. A high score for p53 would indicate the presence of an abnormal p53 protein,

as the normal protein is unstable and not normally detected with immunohistochemical staining techniques. It is accepted however that absent staining in the presence of abnormal p53 can occur if there are truncations in both alleles meaning no p53 protein is produced [163].

The results are shown in table 5.9 below. None of the samples with a wild-type gene sequence stained positive for an abnormal p53 protein as would be expected. Of the samples with a mutant p53 the dl1520 injected samples scored an average of 3.2 +/-0.3 and the saline injected controls scored an average of 2.2 +/-0.5. This difference was not statistically significant when the Wilcoxan rank sum test is used. Some samples with a mutant p53 on gene sequencing did not stain positive for abnormal p53 protein using immunohistochemistry. One explanation for this is that the genetic mutation involved both alleles of the genome, making production of any p53 impossible. It is also possible that if only very small quantities of mutant p53 were produced they might not have been detectable using immunohistochemistry [163]. Most of the normal tissue biopsies did not stain positive for abnormal p53 indicating that they did not have significant amounts of abnormal p53. 5 of 13 biopsies did however stain positive for abnormal p53 with an average score of 2.6 +/-0.3, indicating some defect in p53 production. This was the case in both the normal tissue biopsies that stained positive for the presence of adenovirus.

Table 5.9 - Levels of p53 expression as determined by sample type at all type points combined

Tissue	P53 expression+/- SEM
Normal mucosa overall	1.4+/-0.4
dl1520 injected tumour overall	2.3+/-0.5
dl1520 injected tumour mut p53	3.2+/-0.3
dl1520 injected tumour wt p53	0
Saline injected tumour.	2.2+/-0.5

P53 abnormalities as detected by immunohistochemistry are apparently slightly higher in tumour samples that in the normal tissue as would be expected. This difference was not however significant when the Wilcoxan rank sum test was applied. Some staining for p53 was detected in normal tissue biopsies, possibly indicating some abnormality of p53 function in these tissues.

Results were further analysed to see if there was a higher detection of dl1520 in normal tissue expressing p53 abnormalities compared to normal tissue samples that showed no p53 expression.

Table 5.10 - Viral detection in normal tissue determined by p53 expression

Tissue	ISH fields +/- SEM	ISH density +/- SEM	Hexon fields +/- SEM	Hexon density +/- SEM
P53 negative	0+/-0	0+/-0	0+/-0	0+/-0
P53 positive	1.14+/-0.35	3.5+/-0.3	0.25+/-0.2	0.125+/-0.1

It can be seen that the samples staining positive for p53 in the normal tissue i.e. those with abnormal p53 had apparently higher scores for both hexon adenoviral protein expression and ISH for adenoviral DNA than those not staining positive for p53 i.e. those with normal p53. The only result that showed a significant difference when the Wilcoxan rank sum test was applied however was the ISH value for density (p=0.035).

5.7 - P21 expression – immunohistochemistry

The results of p21 expression were expressed using the same format as p53 expression above. Initially values were compared between tumour samples of either wild-type or mutant p53 status. The results were as follows:

Table 5.11 - P21 expression in tissue samples correlated with p53 status

Tissue	P21 expression +/- SEM
dl1520 injected tumour wild-type	2.75+/-1.1
dl1520 injected tumour mut	3.2+/-1.9
Saline injected tumour wild-type	3.25+/-1.1
Saline injected tumour mut	3.1+/-1.0

As the table shows there was no significant difference in p21 expression either between the wild-type or mutant p53 groups or between the dl1520 injected or saline injected hemi-tumours. The results were further assessed to compare p21 expression at the differing time points for all the different tissue types.

Table 5.12-p21 expression in tissue samples at different time points

Tissue	P21 expression +/- SEM
Tumour 24 hrs	2.4+/-1.4
Tumour 72 hrs	3.6+/-1.0
Tumour 14 days	3.2+/-1.1
Normal tissue 24 hrs	4.2+/-1.5
Normal tissue 72 hrs	4.8+/-1.3

These results show no significant difference between tumour samples or normal tissue samples over the time course. It seems that p21 expression is higher in the normal tissue samples than in tumour samples at all the time points assessed although his does not reach statistical significance. P21 activity is associated with p53 activity as p21 is a major down stream effector of p53. If the virus were triggering a greater apoptotic response in normal tissue compared to tumour tissue this finding would be anticipated.

5.8 - Systemic immune response

Whole blood samples were taken at screening and at the day of surgery and assessed for expression of various immune associated antigens as detailed in materials and methods. An assessment was made of the ratio of CD3/CD4positive to CD4 positive only cells representing the ratio of lymphocytes to monocytes. Each subset of patients from the three different time-points was analysed separately, giving five values in each sub-set. The results are as follows:

Table 5.13 – ratio of lymphocytes to monocytes pre and post viral injection

	Screen Lymphocytes v's Monocytes	Surgery Lymphocytes v's Monocytes
24hrs	1.22 +/-0.16	0.5 +/-0.8
72hrs	2.13 +/-0.17	2.08 +/-0.37
14 days	2.21+/-0.3	1.63+/-0.17

This indicates that at the 24 hour time-point there was a significant drop in the ratio of lymphocytes to monocytes, this being significant $p<0.05$ (Wilcoxon rank sum test). This difference disappears at the later time-points.

An assessment was also made of CD4 positive lymphocytes to CD8 positive lymphocytes. The results are as follows:

Table 5.14 – ratio of CD4 +ve lymphocytes to CD8 +ve lymphocytes pre and post viral injection

	Screen (3+4)v(3+8)+/- SEM	Surgery (3+4)v(3+8)+/- SEM
24	1.97+/-0.66	1.36+/-0.43
72	2.32+/-0.63	1.96+/-0.35
14	4.24+/-1.1	2.02+/-0.41

This indicates a drop in the ratio of 3+4 expressing lymphocytes to 3+8 expressing lymphocytes at all time points although these values did not reach statistical significance.

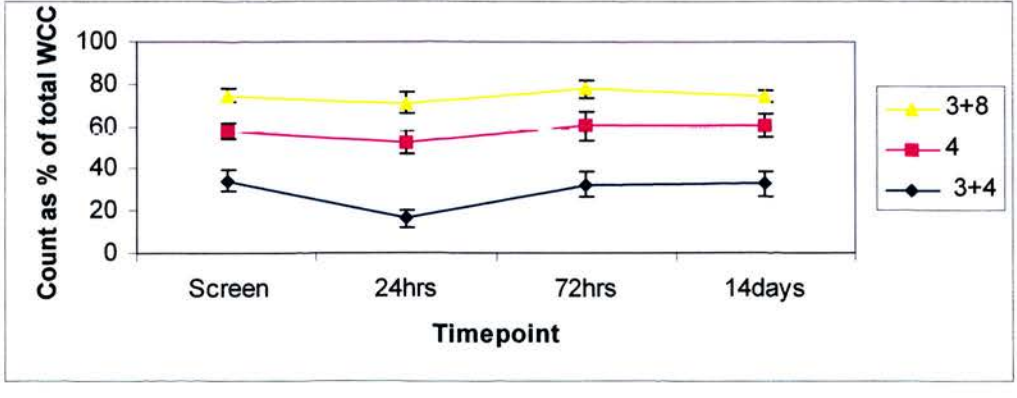
These results were further analysed to see if these changes were due to an absolute drop in the number of CD4 positive lymphocytes or a rise in the number of CD4+ve only cells (monocytes) or CD4/CD8+ve cells. The following graph shows values of the various groups expressed as a % of the total white cell count (WCC). This demonstrates that

there was a fall in the number of CD3/CD4+ve cells and no change in the relative number of monocytes or CD4/CD8+ve cells.

Table 5.15 – ratio of CD4 +ve lymphocytes to CD8 +ve lymphocytes as compared to total number of monocytes pre and post viral injection

	CD 4 +ve lymphocytes (3+4 +ve) % +/- SEM	Monocytes (CD4 +ve) % +/- SEM	CD 8 +ve lymphocytes (3+8 +ve) % +/- SEM
Screen	34.3+/-5.1	23.5+/-3.9	16.8+/-3
24hrs	16.4+/-4.2	36.2+/-5.6	18.4+/-5.3
72hrs	32.4+/-6.1	27.8+/-7.2	17.4+/-4.1
14days	32.8+/-6.0	27.4+/-5.5	14+/-2.8

Figure 5.9- showing the systemic immune response to injection of dl1520



5.10 Summary

The above results can be summarised as follows:

1. Direct intra-tumoural injection of dl1520 is well tolerated in patients with primary intra-oral SCC.

2. In this trial the use of the virus caused no significant side effects.

3. Direct injection of the virus into normal tissue specimens caused no evidence of tissue damage or adverse events.

4. Following direct injection, the virus exists and replicates preferentially in tumour tissue as opposed to normal tissue.

5. It is likely that the virus replicates preferentially in p53 mutant as opposed to p53 wild type tumours, but this was not demonstrated to a level that would show statistical significance in this trial.

6. Injection of the virus into normal tissue appears to stimulate increased apoptosis at the 24 hr time-point. This is in keeping with the proposed mechanism of selective action of the virus i.e. that once inoculated into cells with a functional p53 it would stimulate apoptosis.

7. Localised injection of the virus causes a transient, systemic drop in the CD4+ve lymphocyte count which is manifest at the 24 hr post-injection timepoint.

CHAPTER SIX -

DISCUSSION OF CLINICAL TRIAL RESULTS

6.1 - Pre-operative injection of dl1520

Previous studies have demonstrated the successful use of the virus by direct injection in lymph node metastases in SCC of the head and neck [137, 139]. These lesions were essentially superficial in that the virus was administered trans-cutaneously. This study is the first, in which intra-oral lesions were treated with this virus, which was administered trans-mucosally within the oral cavity.

6.2 - Side effects and adverse events

Previous experience of the virus when administered by direct intra-tumoural injection showed that side effects attributable to the virus such as pyrexia, headache and nausea were recorded quite frequently [137, 139]. In this trial however no significant side effects attributable to the virus were noted during this protocol. This could be explained by the relatively low dose of adenovirus used in this study compared to previous trials and the fact that only a single administration was used. The route of intra-tumoural injection would not be expected to produce a significant systemic viraemia. The patients in this series were also relatively fit and likely to have a more normal immune system function as compared with the subjects in earlier studies who all had advanced, incurable disease. Earlier studies where side effects were more frequent and severe involved high doses and other routes of administration where viraemia was more likely e.g. intra-peritoneal infusion. This is further evidence that the intra-tumoural administration of an E1B deficient, replication competent adenovirus is safe in this group of patients. Other adverse events that were recorded included free flap loss, respiratory failure and transient ischaemic attack. In patients receiving major head and neck surgery these events would be predicted and were not thought to be associated with the adenoviral injection.

Safety is a key issue with all new medicines but particularly with a field such as gene therapy where potentially harmful viruses are used as carriers. Dl1520 is a replication

competent virus meaning that any harmful effect of the virus might be propagated by replication of the virus. There has in the past been one reported fatality of a patient on a gene therapy clinical trial using a replication competent adenovirus where the death was attributed at least in part to the virus being used as a carrier. This trial demonstrates that the dl1520 appears to have a good side effect profile with little risk of adverse events.

6.3 - Clinical effect

6.3.1 - Clinical effect - normal tissue

The apparent effect of the injection of virus into normal tissue was negligible in this trial. This is in keeping with the theory of p53 selectivity for the virus as there would be little or no dysfunction of p53 within normal tissue. Some inflammation might be expected after the injection of adenovirus but if there were little viral replication this would be minimal and short lived, as was seen in this study. Any cell death would be likely to be caused by apoptosis following viral injection in p53 competent tissue and this would be an early, self-limiting event. This fact is of key importance in this study. The main action that the virus is purported to have is based upon selective destruction of tumour cells, in particular cells which have a p53 mutation.

6.3.2 - Clinical effect – tumour

In previous studies direct intra-tumoural injection of the virus into tumours caused measurable tumour shrinkage often associated with tumour necrosis. No tumour shrinkage was recorded in this series despite most of the tumours having p53 mutation and there being evidence of viral replication in many samples. In such samples viral replication leading to cytolysis would be expected with resultant tumour destruction. The fact that this was not noted to a measurable degree is probably because the dose of dl1520 used was relatively low and only a single injection was used. In previous studies

looking at the treatment of recurrent SCC of head and neck where clinical responses were noted doses of up to 10^{11} pfu were used and injections were repeated [139]. It is possible that a higher dose or a multiple dosing schedule would have caused tumour shrinkage in these primary tumour samples.

Previous laboratory and clinical studies have already demonstrated the ability of the virus to cause shrinkage of tumours in both animal model and human studies and this was not the intent of this study (see [75] for review). The most important point when examining the results of this trial at a gross i.e. naked eye level is that the agent did not cause damage to normal tissue despite a direct injection into this tissue. This means that treatment protocols involving systemic or regional as opposed to local administration of the agent might be possible.

6.4 - Histological analyses

6.4.1 - General findings

There was no evidence of gross tissue destruction due to the viral injection when overall tumour size was considered. There was however microscopic evidence of some localised cell lysis in tumour cells positive for adenoviral DNA. This effect was relatively minor and it was not felt that it could be accurately quantified. All of the samples where this was evident had a mutant p53 on gene sequencing. This would support the hypothesis that the dl1520 virus can cause selective tumour cell lysis in p53 negative tumour tissues although the effect was localised and not sufficient in this trial to cause tumour shrinkage. No evidence of lysis was found in the normal tissue biopsies or in tumour biopsies with a wild type p53.

6.4.2 - Dl1520 distribution – immunohistochemistry and in-situ hybridisation

Immunohistochemistry for adenoviral hexon protein demonstrated evidence of viral

presence in 8 out of 15 samples of dl1520 injected tumour. Of these seven had a mutation of p53 and one had a normal p53 sequence. Adenoviral hexon protein was detected in 3 samples of saline injected tumour. All of these had a mutation of p53. In the normal tissue biopsies adenoviral hexon protein was detected in 2 samples. Immunohistochemistry on both of these samples did indicate positive staining for p53 that might indicate a mutation leading to loss of function or of this protein [162, 163, 165].

In-situ hybridisation demonstrated evidence of adenoviral DNA in 6 samples of dl1520-injected tumour. Of these five had a mutation of p53 and one had a normal p53 sequence. Adenoviral DNA was detected in 2 samples of saline injected tumour. Both of these had a mutation of p53 and none had a normal p53 sequence. In the normal tissue biopsies adenoviral DNA was detected in two samples.

These results show that the dl1520 virus is replication competent within human squamous carcinoma even after a single injection of a low viral dose. We have demonstrated a relatively high level of virus detection (11 out of 13 cases altogether when the two detection modalities are combined) compared to earlier trials where a larger dose of virus was used to treat recurrent tumours. In a phase I trial virus was only detected in 4 out of 22 samples and in a phase II trial using dl1520 and chemotherapy virus was detected in 4 out of 10 cases [137, 139]. This could be due to the relatively small tru-cut biopsies obtained from large, heterogeneous tumours in the earlier studies leading to a sampling error rather than being due to increased levels of virus replication in these primary tumours. This is compared to the analyses from this study where sections of entire hemi-tumours were analysed.

There was little evidence of viral survival or replication in p53 wild-type tumours. Only one out of four wild-type tumour showed a positive stain for hexon protein at the 14 day time point and one out of four showed positive staining for adenoviral DNA using in-situ

hybridisation at the 72 hr timepoint. In both cases the virus was only present in 2 high-powered fields in each sample, indicating a relatively small amount of virus in each example.

These findings suggest that the virus can exist preferentially in p53 negative tumour tissue although it can survive in tumours with a normal p53 gene sequence. It was not possible to demonstrate a statistically significant difference between the p53 wild-type and mutant tumour samples however due probably to the small numbers involved. There was however a statistically significant difference between all the tumour samples and the normal tissue samples overall when all the timepoints for analysis were taken together.

The main thrust of the argument for the selective anti-tumour effect of dl1520 is that the specificity is due to p53 function of tissues. This has been demonstrated in several studies looking at both cell line and xenograft models using the virus alone or in combination with other agents. [1, 6, 7, 134]. It is therefore an important point to determine whether the virus can exist and to what extent it can replicate within tissues of each p53 status. Dl1520 has been specifically designed to be replication deficient in p53 competent tissues although this has been a matter of much debate[5] (see below). Our results indicate that the virus has a preference for p53 mut tumour tissue as opposed to either wild-type tumour tissue or normal tissue. The selectivity is not absolute and we did detect small amounts of virus in both p53 wild-type tumour and normal tissue.

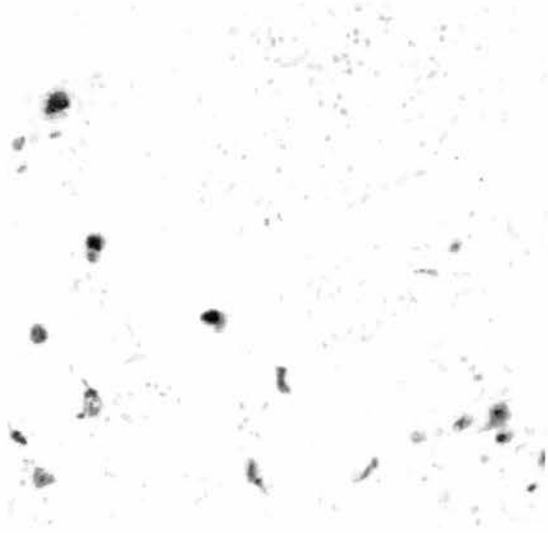
There are several explanations as to why the virus can exist in p53 wild-type tumour samples. The first is to acknowledge the gene sequencing technique used, as with all such techniques, is not certain to pick up all the mutations present in any specific gene. It is not impossible therefore that the two samples that showed evidence of viral replication harboured mutations, which were not detected by the gene sequencing. Another possibility is that even with a wild-type gene sequence for p53 the entire cycle of p53 function could be disturbed by mutations in other important cell cycle regulator genes

such as p21, p19 or p16 [9, 111, 166]. It is possible therefore that the function of p53 as the key regulator of apoptosis following viral inoculation as a whole could be disrupted by factors other than the p53 itself [25].

Another explanation is the ability of the dl1520 virus to exist and even replicate within p53 functional tissue. Several papers have demonstrated the ability of the virus to exist and replicate within p53 competent tumour cells in both a tissue culture and tumour xenograft model [1, 126]. According to the theory of the select action of the virus it should be able to exist in p53 competent tissues and even cause limited cell death due to apoptosis. It is salient that in the p53 wild-type samples where virus was detected there was no evidence of cytolysis. If the virus could exist in tissue with a fully functional p53 protein it would be expected that in these wild-type tumours with viral presence there would be an excess of apoptosis. This was not found in this study although the small number of cases that involved tumour with wild-type p53 showing evidence of virus makes any comparisons difficult.

The virus was usually confined to the injected hemi-tumour with little evidence of viral spread to the saline injected control. Viral DNA was however detected in two saline injected samples. This could either be due to viral spread throughout the tumour or to inadvertent viral injection in the control tumour. The hemi-tumours were marked carefully with tattooing of blue dye at the time of injection and great care was taken to sample the correct areas once the tumours were resected and tissues were processed to prevent this sampling error. The virus detected within the saline control samples was not confined to the midline of the tumours and so it is likely that this effect is due to spread of the virus. There was however little evidence of tumour spread with only two samples showing this phenomenon. This implies that following a single injection the dl1520 is capable of spread throughout a tumour but that this is a limited phenomenon.

Figure 6.1 - Viral DNA in saline injected control sample



There are many factors that could act to prevent the spread of virus throughout a tumour. These include intra-tumoural pressure gradients, extracellular matrix molecules and immune cells [167]. The fact that there was some evidence of viral spread into non-injected tumour tissue after a single injection is encouraging and indicates that some spread can take place. In total virus was detected in two saline injected samples by ISH and in three saline injected samples by immunohistochemistry. All of the samples demonstrated a mutant p53 sequence. Virus was not detected in any saline injected samples with a wild-type p53 gene sequence. This would suggest that any distribution through tumour not directly injected with the virus occurs preferentially in p53 null tissue. Recent work involving dl1520 in a human cell murine xenograft model has previously demonstrated that distribution is related to p53 status in this model [132].

It was also noted that other factors concerning the method of injection could act to increase distribution of virus, namely dividing the given dose of virus into several injections and increasing the volume of carrier injected. Increasing the distribution of virus in this way leads to increased anti-tumoural efficacy in this experimental model [132]. As the volume of carrier as related to the volume of tumour mass was constant in this trial, at 20% of the tumour volume, this factor should have been the same in all cases.

6.4.3 - Detection of dl1520 in normal tissue

Three biopsies of normal tissue injected with dl1520 demonstrated either presence of hexon protein or adenoviral DNA. In two cases this was at the 24 hr timepoint and one case at 72 hrs. There was no evidence of tissue destruction in these biopsies. All three of these samples demonstrated positive immunohistochemical staining for p53 protein, indicating that the protein was abnormal in some way. Gene sequencing was not performed on the normal tissue biopsies but it is indicated by the positive staining that there was probably some abnormality of function in these samples. It is not surprising

that dl1520 can survive for a limited period in normal tissues, as this would be predicted by its proposed mechanism of action. The virus would be predicted to cause apoptosis in infected normal tissue within a relatively early timescale and would not be expected to replicate and cause cytolysis. Any tissue damage would be minor as little viral replication or cytolysis would be expected. This is borne out by the fact that no gross changes or ulceration was noted in the samples and there was no microscopic evidence of tissue damage.

These results would refute recent suggestion that the dl1520 virus can replicate independently of the p53 status of host cells. It has always been known that the virus can exist and replicate to a certain extent in p53 wild-type tissues of both tumour and normal tissue although this is very restricted compared to levels of viral replication in p53 mut tumour cell lines [1, 5]. It is also clear that the virus survives selectively in tumour tissue as opposed to normal tissue following direct injection.

Recent publications have questioned this selectivity of action of the virus. Some workers have suggested that the p53 selectivity of the virus is dose dependent, with p53 selectivity at low doses which is lost when higher doses of the virus are used [128]. This loss of selectivity is proposed to be due to the fact that if sufficient adenovirus is present in combination with sufficient cell numbers to be infected the virus is not dependent on stimulating cells to enter S-phase. This means that the p53 status of cells is not crucial to viral survival. Other workers have suggested that there are both p53 dependent and independent factors required for the replication of the dl1520 adenovirus [88]. This paper confirmed that a functional p53 does restrict the level of dl1520 replication but that other factors were also involved. These other factors are thought to exist because the E1B protein deficient in this virus is not just involved in inactivating the p53 protein but also in the manufacture of late viral mRNA. The level of this dependence on E1B for the manufacture of mRNA seems to vary between host cell lines in a manner independent of p53 status.

Other factors which can play a part in determining the effectiveness of cytolytic viral therapy in a given cell line include the infectivity and subsequent expression of early viral proteins. This has recently been shown to vary between different human tumour cell lines in a tissue culture model which again can explain why the effectiveness of the virus can depend on factors other than p53 status of tissues [129].

Hall et al recently published results from tissue culture experiments suggesting that the virus even required functional p53 to kill tumour cells [126]. This is in direct contradiction to both the theoretical situation and the findings of most workers in this field. It is noteworthy that these workers used only a tissue culture model and did not use exactly matched cell lines i.e. cell lines identical except for p53 function. They also analysed results at a relatively early stage when viral replication may not have been as important as initial viral infection. As shown above there are many factors which could potentially influence the ability of dl1520 to replicate and cause cytolysis so the only way to demonstrate p53 functional requirements accurately is to use matched cell lines.

Other authors have also described results indicating that the virus can replicate within cell lines, both cancer and primary cultured strains, although confirming that this effect is often p53 dependent in the degree of replication [88]. These findings are quite different to those initially reported by Bischoff et al 1996 and Heise et al 1997. Both these original papers reported evidence from multiple tissue culture and murine xenograft experiments to suggest that the virus was selective for replication and cytolytic effect in p53 mut tumour cell lines.

The conflicting evidence from these papers suggests that under certain circumstances the virus can exist and to a greater or lesser extent replicate within p53 wild-type tumour cell lines and some normal cell lines. As most of the results above are from tissue culture experiments only they do not necessarily reflect the situation when the virus is used in a

clinical setting. It is not clear of the significance of viral detection in normal and p53 competent tissue. So long as there is no evidence of harm to normal tissue the fact that a small amount of virus can be detected in biopsies does not rule out the safety of the virus as a treatment for tumours.

The goal of this form of gene therapy would be systemic delivery of virus rather than intra-tumoural injection. This would allow microscopic, undetectable deposits of tumour to be treated with the virus along with any clinically obvious disease. It has been demonstrated that intra-venous viral delivery is feasible and causes tumour regression in a murine xenograft model using a p53 mut human tumour cell line. If this were to be repeated in humans the virus would have to be selective for tumour cells with little risk of damage to normal tissue. Our study has demonstrated that even after a direct injection of virus into normal tissue no damage to the tissue was demonstrated. The fact that virus was detected in a small number of such tissue samples does not mean that the cytolytic activity of the virus is not selective. We have demonstrated that any cytolysis does seem to be selective for p53 mut tumour tissues. This would encourage the possibility of dl1520 being a possible agent for systemic gene therapy of human tumours.

6.4.4 - Apoptosis

Tunel staining was used to investigate the mechanism of cell death within injected samples. The results indicated that apoptosis was occurring within both the saline injected tumour samples and the viral injected tumour samples. In the dl1520 injected samples there was an initial high level of apoptosis at 24 hrs, which quickly tailed off by 72 hrs. The saline injected samples did not have this initial high level but this difference was not statistically significant between the two. A relatively high level of apoptosis was noted in the normal tissues at the 24 hr timepoint and this value was statistically significantly higher that in the saline injected sample. This indicates that at 24 hrs the

dl1520 stimulate apoptosis in both normal tissue and tumour samples. In the normal tissue this apoptosis persists until at least 72 hrs whereas in the tumour sample the apoptosis level comes down by 72 hrs. If it had been possible to obtain baseline biopsies from the normal tissue this would have given very useful information to indicate if these differences were real or due to chance, but consent for these additional biopsies was not obtained for this trial.

It has been previously noted that the dl1520 virus can kill tumour cells via apoptosis in a tissue culture model and that this effect is increased in p53 competent cells [136]. It is also well recognised that intra-oral SCC can undergo apoptosis in response to chemotherapeutic regimens [168]. Dl1520 is purported to destroy p53 null tumour cells principally by direct cytolysis. P53 wild-type cells should respond to inoculation with dl1520 by undergoing apoptosis. In this study no difference in levels of apoptosis between dl1520 injected hemi-tumours or saline injected was noted when all time points were assessed. If the 24hr delay samples were taken in isolation however there was an increase in apoptosis between the virus and saline injected samples although this did not reach significance.

This is in keeping with the previously noted fact that dl1520 can stimulate some apoptosis even in p53 mutant tumour cells. As none of the 24hr delay samples had a wild-type p53 configuration it is impossible to say whether a wild-type sample would have had higher levels of apoptosis. This effect seems to be lost at later time-points with no difference between the groups at 24hrs or 72 hrs. This could be explained if the virus stimulated a burst of apoptosis in susceptible cells following inoculation. Following this those cells susceptible to this cell death would die in a controlled fashion, limiting local virus spread. This effect would be short lived.

There was also no significant difference in apoptosis levels between p53 mutant or wild type tumours. As few wild-type tumours were assessed, and all of these at the later time-

points, it is hard to draw any conclusions from this. In theory the virus should stimulate relatively high levels of apoptosis in p53 wild-type tumours due to the action of the viral E1A protein stimulating DNA synthesis which is a trigger to p53 mediate apoptosis [119]. This effect would be expected soon after injection and as none of the samples with wild-type sequence were from the 24hr group it may simply have been missed in these samples as they were sampled after the effect had rescinded.

Increased apoptosis was noted in the normal tissue biopsies at all the time-points assessed. It would be expected that injection of the virus into normal tissues with a functional p53 dependent apoptotic pathway would stimulate apoptosis to a relatively high level. As before the action of the E1A viral protein would lead to p53 mediated apoptosis in these tissues. This effect would lead to a containment of the viral infection with little viral replication and this was seen in this trial as by day 14 the level of apoptosis had decreased.

The above findings are supportive of the concept that dl1520 can induce high levels of apoptosis in normal tissues. It also suggests that apoptosis in tumour tissues, including those with mutant p53, can be stimulated. In the wild-type adenovirus the E1B protein blocks apoptosis via the p53 pathway. It has also been suggested that E1B can also block apoptosis mediated by the Bax protein. This protein is known to bind the E1B 19K protein and it is thought that this is another mechanism by which an adenovirus can prevent apoptosis of the host cell [119]. The Bax protein has been demonstrated to be able to induce apoptosis independently of p53 and it is possible that in the p53 mut tumours some apoptosis could have been induced via this pathway.

It is clear that just as p53 can lead to apoptosis via several different stimuli so apoptosis can be triggered via other mechanisms that do not involve p53 [169]. It is known for example that in oral carcinoma cells collected in India over expression of the heat shock HSP70 protein acts to block apoptosis of tumour cells [118]. This could explain the fact

that apoptosis was not noticeably higher in p53 wild-type tumours as compared to p53 mut tumours.

6.4.5 - Immunohistochemistry for p53/p21

Immunohistochemical staining for p53 demonstrated a good correlation between staining and gene sequencing. The four wild-type tumours on gene sequencing all stained negative for p53, indicating a normal p53 protein that, due to its short half-life, is not normally detectable by immunohistochemistry. This indicates that the immunohistochemical results correlated with the gene sequencing results. Of the tumour samples with a mutant p53 on sequencing 8 out of 10 stained positive for p53.

There are several possible explanations for this difference between the two detection methods. It is known that methods of tumour fixation can affect the stability of p53 protein which could affect the p53 staining pattern [163] . It is also possible that tumour harbouring a mutation causing both alleles of p53 to be lost would not produce any p53 or insufficient to be detected. It is also relevant that the area biopsied for Genechip analysis was by necessity different from that assessed by immunohistochemistry, even though the areas would have been close together. This may have affected the immunohistochemistry pattern as compared to the p53 sequencing [165]. As noted in the materials and methods section, the Genechip analysis cannot be considered 100% accurate. Any of the above factors could have contributed to the discrepancy between gene sequencing and immunohistochemistry for p53.

There was no significant difference in p53 detection between the dl1520 injected tumour samples and the saline injected. This was true at all the timepoints assessed. This would indicate that the injection of dl1520 virus was not stimulating p53 protein to be expressed in these tumours. This would be expected as those tumours with a mut p53, which is dysfunctional, would not be expected to respond to the injection of adenovirus. In the wild-type cases where p53 protein would have been stimulated none was detected,

as immunohistochemistry tends to detect only abnormal p53.

The normal tissue samples showed little staining with p53 as would be expected in non-tumour tissue. 8 out of 15 samples did stain for p53 indicating a degree of abnormality of the protein within these tissues. It is well known that smoking and drinking can lead to overexpression of p53 in the mucosa adjacent to SCC of the head and neck [90, 91]. All the mucosal biopsies of normal tissue were taken from grossly normal buccal mucosa distinct from the tumours. All the patients sampled were known smokers. It is not surprising therefore that some overexpression of p53 was noted in these samples. It is likely that such overexpression is due to mutations in the p53 gene and therefore loss of function of the p53 protein as has been demonstrated in other studies [170] [4]. It is noteworthy that in the three samples of normal tissue where virus was detected there was also staining for p53, indicating a possible loss of function of this protein. This would help explain the survival of the dl1520 virus within these normal tissue samples although not all the samples staining positive for p53 had evidence of viral survival.

P21 expression was found in all samples both the tumour and normal tissue and in wild type and mut p53 tumour samples. The level of p21 expression did not vary between the dl1520 and saline injected tumours indicating that p21 was not preferentially stimulated by the addition of the adenovirus. P21 is thought to be one of the main downstream effector of p53 and expression of p21 would be expected to correlate with the activity of p53 within the cell. Overall this is in keeping with the immunohistochemical findings of this project as there was no difference between expression of p53 between dl1520 and saline injected tumour samples. What is more surprising is the fact that p21 expression in p53 mutant tumour samples was the same as that for wild-type samples. It might have been predicted that there would be less p21 expression in p53 mutant tumours, as there was little or no functional p53 to stimulate the formation of p21.

P21 has however been shown to be induced by p53 independent pathways however and

could be acting in this case independently of p53 [112]. P53 can also act independently of p21 to cause apoptosis [25] and so it could be expected that p21 can function and be expressed independently of p53.

In the normal tissue samples levels of p21 were relatively high at all time points following injection of the virus. This correlates with the level of apoptosis in the samples, which was high compared to that found in the tumour samples. This would be in keeping with the theory of the dl1520 adenovirus causing apoptotic cell death in normal tissue. It can be surmised that p21 is playing a part in this apoptotic cell death. There was no significant difference between the different timepoints. This was unlike the levels of apoptosis however, which seemed to decrease at the 14-day timepoint. We cannot therefore be certain that p21 expression is increased in normal tissue samples following viral injection but we have produced some evidence to support this hypothesis.

6.5 - Systemic immune response

FACS analysis of peripheral blood samples taken at screening and immediately prior to surgery were analysed to look at the effect on the immune system of direct intra-tumoural injection of the adenovirus. The main finding of this was that we have demonstrated a fall in the ratio of lymphocytes to monocytes and also a fall in the ratio of CD4 positive lymphocytes to CD 8 positive lymphocytes. This finding has not previously been documented following an intra-tumoural administration of viral therapy. The changes in the peripheral blood picture are transient and disappear after 72 hrs. The cause of these effects is not at present clear. Possible explanations might be sequestration of CD4 positive lymphocytes in to the injection sites or possibly the destruction of the lymphocytes in the peripheral blood. This phenomenon has not been previously described in the literature. It is unclear if this reaction was related solely to the administration of adenovirus or was related to the effects of surgery. These findings

would merit further investigation in any future clinical trial.

This drop in CD4+ve lymphocytes did not seem to affect the patients clinically in any way. There was no evidence of increased risk of wound or other infections and no increase in complications compared to what would have been expected in the normal course of events. The significance of this transient alteration in the peripheral blood count remains to be determined.

CHAPTER SEVEN - CLONOGENIC ANALYSIS OF CELL LINES A2780 AND A2780CP70 IN COMBINATION WITH DL1520 AND CHEMOTHERAPY AGENTS

Clonogenic Analysis Of Cell Lines A2780 And A2780CP70 In Combination With Dl1520 And Chemotherapy Agents

7.1 - Materials and methods

A tissue culture model was established to determine the effect of dl1520 adenovirus in combination with chemotherapeutic agents in two cells lines matched for p53 status. The virus was incubated for either 24hrs or 72 hrs and the cells were then treated with chemotherapeutic agents in varying concentrations. Clonogenic analysis was then carried out to determine the number of viable colony forming cells left following this treatment[20]. All assays were performed three times.

7.1.1 - Cell lines

Cell lines were derived from the ovarian carcinoma cell lines A2780 and A2780CP70. These cell lines are matched for p53 status with A2780 having a wild-type p53 function whereas A2780CP70 has been treated to have dysfunctional p53 [171] [19]. This p53 resistance has been established due to repeated treatments with the DNA damaging agent cisplatin. The A2780 cell line is a cisplatin sensitive cell line. The A2780CP70 variant has a greater monolayer growth rate and is 7.3 fold more resistant to cisplatin. This resistance is related to dysfunction of the p53 tumour suppressor protein. These paired cell lines are frequently used to investigate new potential drugs for use in drug resistant tumours.

The cells were maintained in RPMI medium (anti-biotic free) supplemented with 10% fetal calf serum. Cells were maintained in 5% CO2 at 37°C and screening for mycoplasma was undertaken monthly and any cell lines found to be positive were discarded. No additional studies were made to determine the mechanism of cell death within these assays.

7.1.2 - Effect of incubation of dl1520 on A2780 and A2780CP70 in combination with cisplatin

Cultures of both cell lines were trypsinised and suspended in medium. 5 X 10^5 cells were added to 10mm petri dishes each containing 10ml of medium and the dishes were incubated for 24 hrs at 37°C in 5% CO2. The medium was aspirated and the cells in each dish were washed with PBS, trypsinised using trypsin diluted 1:10 and resuspended in medium and a cell count was made to determine the approximate number of cells per dish. The virus was supplied in a concentrated form with the concentration expressed a number of plaque forming units (PFU) per ml stated, which was determined by plaque assay using HeLa cells as previously described [5]. Medium was aspirated from all dishes and the cell monolayers were treated with either 500 µl PBS, 10 PFU/ cell dl1520 in 500µl PBS or 50 PFU/ cell dl1520 in 500µl PBS. The plates were incubated at 37°C in 5% CO2 for 90 minutes with regular mixing of the PBS every 15 minutes.

Non-absorbed virus was aspirated from each plate and 10 ml medium was added to each. The plates were then incubated at 37°C in 5% CO2 for either 24 hrs or 72 hrs. Medium was then aspirated from each plate and replaced with cisplatin diluted in 10ml medium at various concentrations. For A2780 concentrations of 0 µM, 1 µM, 5µM, 10µM and 20 µM were used and for A2780CP70 concentrations of 0 µM, 10 µM, 20µM, 40µM and 80 µM were used. This higher dose was used, as the A2780CP70 is known to be relatively resistant to the action of cisplatin. Plates were incubated for one hour at 37°C in 5% CO2. Medium was again aspirated and the cell monolayers washed in PBS. The cells were then trypsinised and counted. For each variable of virus concentration and cisplatin concentration 10^3 cells were added to 10 ml medium in a 90 mm petri dish in multiples of five for each value. Cells were incubated at 37°C in 5% CO2 for 11 days to allow colony formation. At this time colonies were washed in PBS, fixed in methanol and stained for ten minutes with crystal violet. Colonies were then counted using the colony counter and results expressed as a percentage of surviving fractions compared to

the control i.e. no virus and no cisplatin.

7.1.3 - Effect of incubation of dl1520 on A2780 and A2780CP70 in combination with topotecan

Cultures of both cell lines were trypsinised and suspended in medium. 5 X 10^5 cells were added to 10mm petri dishes each containing 10ml medium. The plates were incubated for 24 hrs at 37°C in 5% CO2. The medium was aspirated and the cells in each dish were washed, trypsinised and resuspended in medium. A cell count was made to determine the approximate number of cells per dish. Medium was aspirated from all dishes and the cell monolayers were treated with either 500 µl PBS, 10 PFU/ cell dl1520 in 500µl PBS or 50 PFU/ cell dl1520 in 500µl PBS. The plates were incubated at 37°C in 5% CO2 for 90 minutes with regular mixing of the PBS every 15 minutes.

Excess virus was aspirated from each plate and the monolayers recovered with 10 ml medium. The plates were then incubated at 37°C in 5% CO2 for either 24 hrs or 72 hrs. Medium was then aspirated from each plate and replaced with topotecan at various concentrations in 10 ml medium. For both cell lines concentrations of 0nM, 50nM, 100nM, 200nM and 40nM were used. Plates were incubated for four hours at 37°C in 5% CO2. Medium was again aspirated and the cell monolayers washed in PBS. The cells were then trypsinised and counted. For each variable of virus concentration and cisplatin concentration 10^3 cells were added to 10 ml medium in a 90 mm petri dish in multiples of five for each value.

Cells were incubated at 37°C in 5% CO2 for 11 days to allow colony formation. At this time the colonies were washed in PBS, fixed in methanol and stained for ten minutes with crystal violet. Colonies were then counted using the colony counter and results expressed as a percentage of surviving fraction compared to control i.e. no virus and no

topotecan.

7.2 - Results

7.2.1 - Clonogenic analysis of the action of d l1520 in combination with cisplatin and topotecan

Two cell lines matched for p53 status, A2780 (wild-type p53) and A2780 CP70 (mutant p53) were used to determine the effect of standard chemotherapeutic agents in combination with dl1520 [171]. The experiments were conducted following different incubation times with the virus. Cells were incubated with the dl1520 virus at concentrations of 0, 10 or 50 pfu per cell for either 24 or 72hrs. These time-points were used as viral replication would be near maximal after 72 hrs so at this time point effects due to replication would be most evident [79]. At 24 hrs any effect due to apoptosis alone would be more likely to be apparent as apoptosis is typically triggered by this time following adenoviral inoculation [9]. Following the incubation period the cells were treated with various concentrations of the chemotherapeutic agent under investigation for a given time, one hour in the case of cisplatin and four hours in the case of topotecan. Following treatment with the dl1520 virus the cells were plated out for a clonogenic analysis to determine the number of colony forming units that survived the treatment.

7.2.2 - Effect of Dl1520 alone with A2780 and A2780CP70

It was found that the dl1520 virus has the capacity to kill cancer cells from both cell lines tested i.e. with wild type or dysfunctional p53 in the absence of any chemotherapy agent. The level of cell kill increased with higher inoculation of dl1520 in the absence of other agents. After 24hr virus exposure the % colony survival dropped from 67% at 10 pfu/ cell to 62% at 50 pfu/ cell for the A2780 and from 94% at 10 pfu/ cell to 66% at 50 pfu/ cell for the A2780CP70. At the 24 hr time-point therefore there is no increase in cell

death in the A2780CP70 cells as compared to the A2780. In fact the opposite is demonstrated, with less cell death in the A2780CP70 with the higher dose of virus at 24 hrs. At this point apoptosis rather than cytolysis would be the predominant mode of cell death caused by the virus and so these findings are not unexpected.

After 72 hr exposure the % cell survival was 70% at 10 pfu/ cell and 70% at 50 pfu/ cell for the A2780 and from 28% at 10 pfu/ cell to 12% at 50 pfu/ cell for the A2780CP70 See fig 3.1 and 3.2). It is clear therefore that in the p53 mutant A2780CP70 cells there is a substantial increase in cell death following a longer incubation with the virus. At the 72 hr timepoint cytolysis, following viral replication, would be the main mechanism of cell and so a cell line permissive for viral replication would demonstrate increased susceptibility to death caused by the virus.

7.3.1 - Effect of the addition of cisplatin with dl1520 to A2780 and A2780CP70 cell lines

The effect on cell death caused by the virus is enhanced by the addition of cisplatin in a dose dependant manner in both cell lines as shown below. As would be expected a higher dose of cisplatin is required in the A2780CP70 as opposed to the A2780 to obtain a similar level of cell kill, due to the lack of functional p53 causing resistance to cisplatin in the A2780CP70 cells.

Adding cisplatin alone to the A2780 cell line causes colony survival to drop from 78% with1μM cisplatin to 10% with 20μM cisplatin. With the addition of 10pfu/ cell of dl1520 with 24 hr incubation with the virus the survival drops to 52% and 7 % respectively for 1μM and 20 μM (see fig 3.3). The addition of 50pfu/ cell of dl1520 with 24 hrs incubation causes the colony survival to drop to 47% and 6 % respectively for 1μM and 20 μM cisplatin. The combination of 72hr incubation with dl1520 along with cisplatin shows that with the addition of 10pfu/ cell of dl1520, colony survival drops to

70% with 1μM cisplatin and to 4% with 20μM of cisplatin. When the viral concentration is increased to 50 pfu/cell the values for colony survival are 70% for 1uM cisplatin and 2% for 20μM cisplatin respectively.

In the A2780 CP70 cell line, adding cisplatin alone causes colony survival to drop from 96% with20μM cisplatin to 29% with 80μM cisplatin. As noted above a larger concentration of cisplatin is required with this cell line to achieve a similar colony survival as compared to the A2780 cell line. Incubation for 24 hrs with 10pfu/cell dl1520 causes the cell survival to drop to 83% with 20μM cisplatin and 16% with 80μM cisplatin. Incubation for 24 hrs with 50pfu/cell dl1520 causes the cell survival to drop to 48% with 20μM cisplatin and 9% with 80μM cisplatin.

Incubation of the A2780CP70 cell line for 72 hrs causes a different level of cell death. With 10pfu/cell dl1520 causes the cell survival to drop to 12% with 20μM cisplatin and 1% with 80μM cisplatin. Incubation for 72 hrs with 50pfu/cell dl1520 causes the cell survival to drop to 6% with 20μM cisplatin and 0.5% with 80μM cisplatin.

It can be seen from the above results that increasing the length of exposure to dl1520 has a different effect on each cell line. After 24 hr exposure to 50 pfu of the virus the difference in effect of dl1520 between the two matched cell lines is minimal with a 62% survival with the A2780 and a 55% survival with the A2780CP70. After 72hr exposure however there was a marked increase in cell killing activity in the p53 null A2780CP70 compared to the wild-type A2780 with a 70% survival with the A2780 and a 12% survival with the A2780CP70.

A similar pattern was found using 10pfu of virus with survival of 67% at 24hrs with A2780 and 94% with A278CP70 but 70% as opposed to 28% following 72hrs viral exposure. It would appear that the differential in viral killing between cell lines of varying p53 status is time dependent. This is in keeping with the proposed mechanism of

action of dl520, as the differential activity would rely on viral replication to disseminate further virus following cytolysis. At 24 hrs however there would not have been sufficient time for this effect to be manifest.

Figure 7.1 – Effect of incubation of A2780 cells with dl1520 at various concentrations for 24 or 72 hours

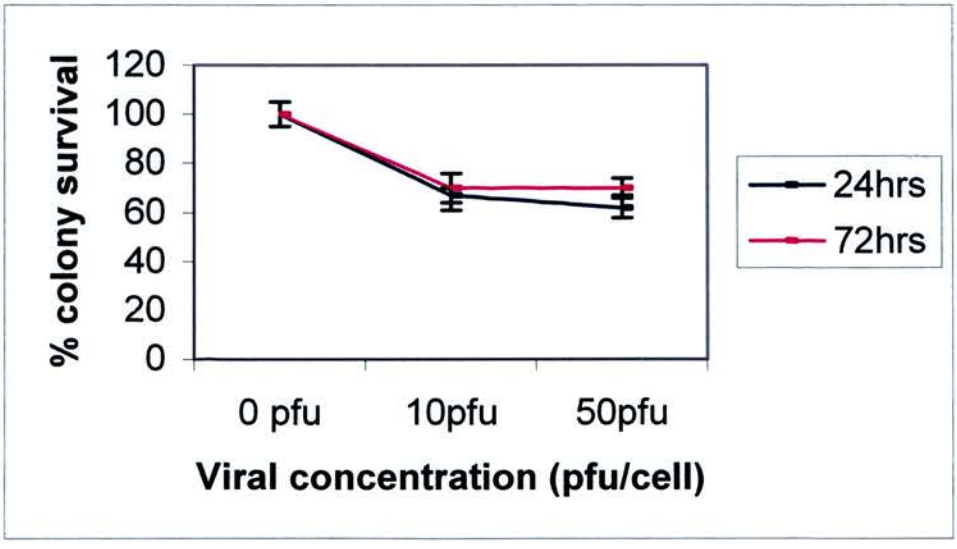


Figure 7.2 – Effect of incubation of A2780CP70 cells with dl1520 at various concentrations for 24 or 72 hours

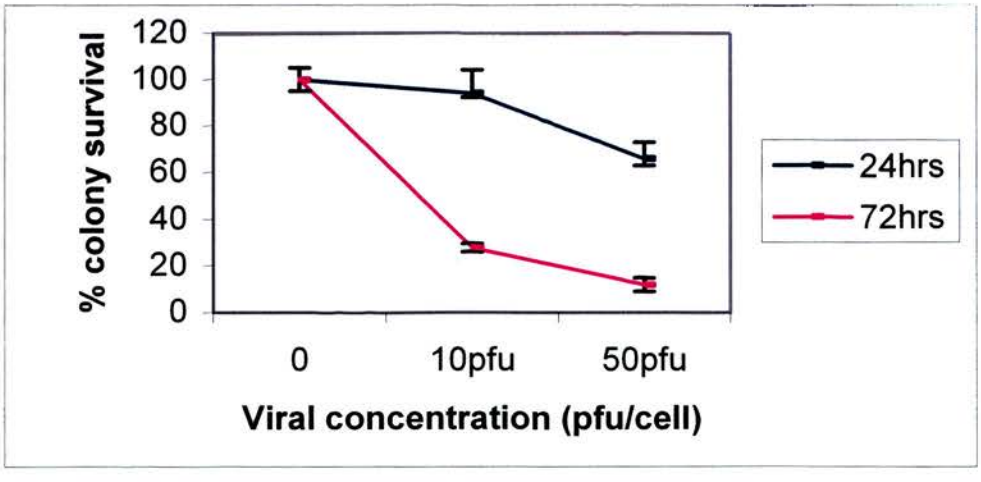


Figure 7. 3 – The effect of a combination of cisplatin + 24hr exposure to dl1520 in the A2780 cell line

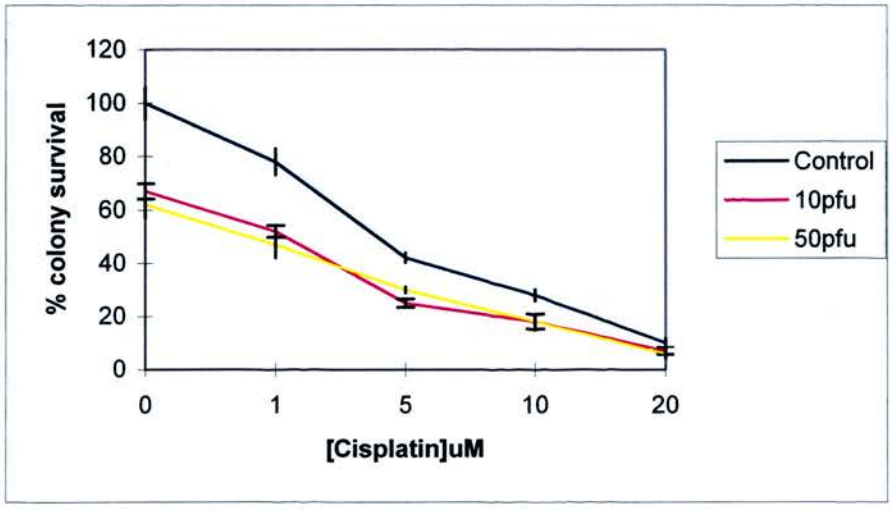


Figure 7. 4 – The effect of a combination of cisplatin + 72hr exposure to dl1520 in the A2780 cell line

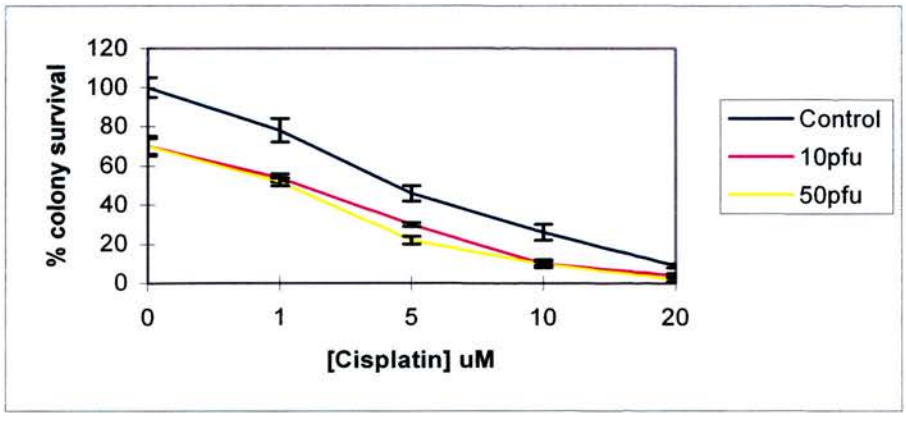


Figure 7. 5 – The effect of a combination of cisplatin + 24hr exposure to dl1520 in the A2780CP70 cell line

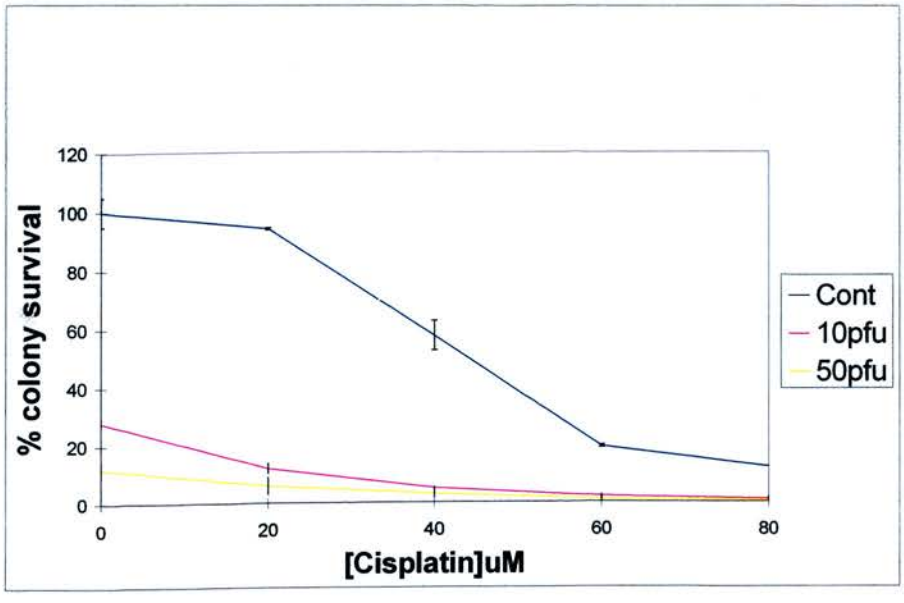


Figure 7. 6 – The effect of a combination of cisplatin + 72hr exposure to dl1520 in the A2780CP70 cell line

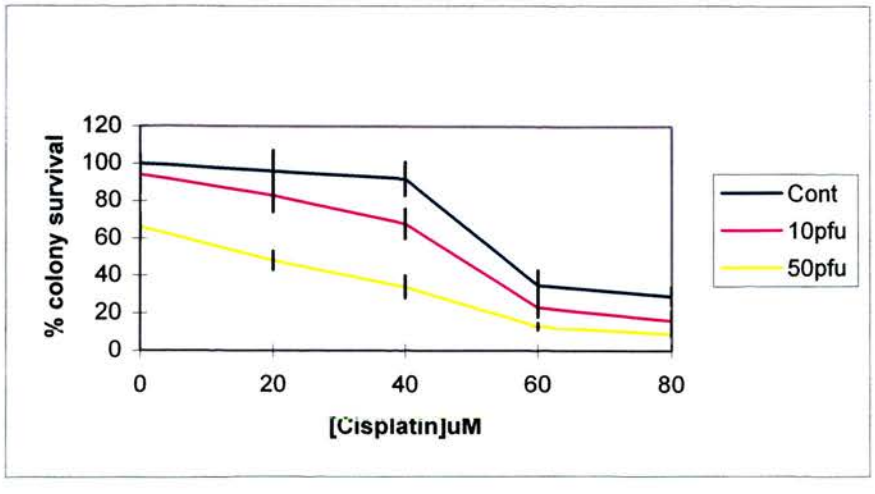


Table 7.1 Clonogenic survival of A2780 cells following 24 hr exposure to dl1520 and exposure to cisplatin as shown. Values expressed as % of control value

[cisplatin] uM	Control +/- SEM	10pfu +/- SEM	50pfu +/- SEM
0	100+/-6	67+/-3	62+/-5
1	78+/-5	52+/-2	47+/-5
5	42+/-2	25+/-1	30+/-1
10	28+/-2	18+/-3	18+/-3
20	10+/-2	7+/-1	6+/-2

Table 7.2 Clonogenic survival of A2780 cells following 72 hr exposure to dl1520 and exposure to cisplatin as shown. Values expressed as % of control value

[cisplatin] uM	Control +/- SEM	10pfu +/- SEM	50pfu +/- SEM
0	100+/-5	70+/-5	70+/-4
1	78+/-6	54+/-2	52+/-2
5	46+/-4	30+/-2	22+/-2
10	26+/-4	10+/-2	10+/-1
20	9+/-1	4+/-1	2+/-1

Table 7.3 - Clonogenic survival of A2780CP70 cells following 24 hr exposure to dl1520 and exposure to cisplatin as shown. Values expressed as % of control value

[cisplatin] uM	Control +/- SEM	10pfu +/- SEM	50pfu +/- SEM
0	100+/-5	94+/-10	66+/-7
20	96+/-11	83+/-9	48+/-5
40	92+/-9	68+/-8	34+/-6
60	35+/-8	23+/-5	13+/-2
80	29+/-4	16+/-5	9+/-1

Table 7.4 Clonogenic survival of A2780CP70 cells following 72 hr exposure to dl1520 and exposure to cisplatin as shown. Values expressed as % of control value

[cisplatin] uM	Control +/- SEM	10pfu +/- SEM	50pfu +/- SEM
0	100+/-5	28+/-2	12+/-3
20	94+/-1	12+/-2	6+/-3
40	57+/-5	5+/-1	3+/-1
60	19+/-1	2+/-1	1+/-1
80	12+/-0	1+/-1	0.5+/-1

7.4 – Effect of the addition of topotecan and dl1520 to the cell lines A2780 and A2780CP70

The above experiment was repeated using topotecan as the chemotherapeutic agent with concentrations of 0, 50, 100, 200 and 400nM of the drug in solution. Results again showed that dl1520 has the capacity to kill cancer cells from both cell lines i.e. with wild type or mutant p53. These experiments gave a pattern of results similar to the previous experiment using a combination of dl1520 virus plus cisplatin. A higher level of cell kill was demonstrated with increasing inoculation with dl1520 in the absence of topotecan. The level of cell kill increased from a % colony survival of 77% at 10 pfu/ cell to 70% at 50 pfu/ cell for the A2780 and from 79% at 10 pfu/ cell to 45% at 50 pfu/ cell for the A2780CP70 after 24hr virus exposure. After 72 hr exposure the % cell survival was 78% at 10 pfu/ cell to 48% at 50 pfu/ cell for the A2780 and from 43% at 10 pfu/ cell to 22% at 50 pfu/ cell for the A2780CP70. This effect is enhanced by the addition of topotecan in a dose dependant manner in both cell lines as shown in figure 7.1.

Increasing the length of exposure to dl1520 has a different effect on each cell line. After 24 hr exposure to 50pfu of the virus the difference in effect of dl1520 between the two matched cell lines is minimal with a % survival of 70% with the A2780 and a % survival of 45% with the A2780CP70. After 72hr exposure however there was a marked increase in cell killing activity in the p53 null A2780CP70 compared to the wild-type A2780 with a % survival of 48% with the A2780 and a % survival of 22% with the A2780CP70. A similar pattern was found using 10pfu of virus with survival of 77% at 24hrs with A2780 and 79% with A278CP70 but 78% as opposed to 43% following 72hrs viral exposure. These patterns were repeated using various concentrations of topotecan as shown by the following graphs.

Figure 7.7 – The effect of a combination of topotecan + 24hr exposure to dl1520 in the A2780 cell line

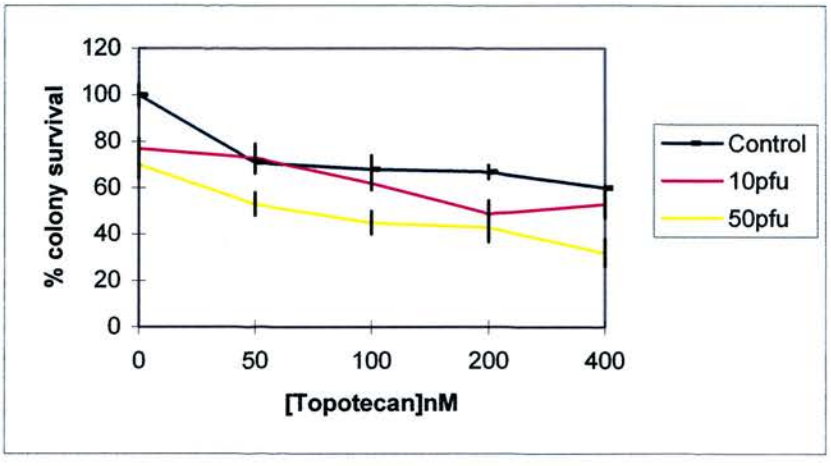


Figure 7.8 – The effect of a combination of topotecan + 72hr exposure to dl1520 in the A2780 cell line

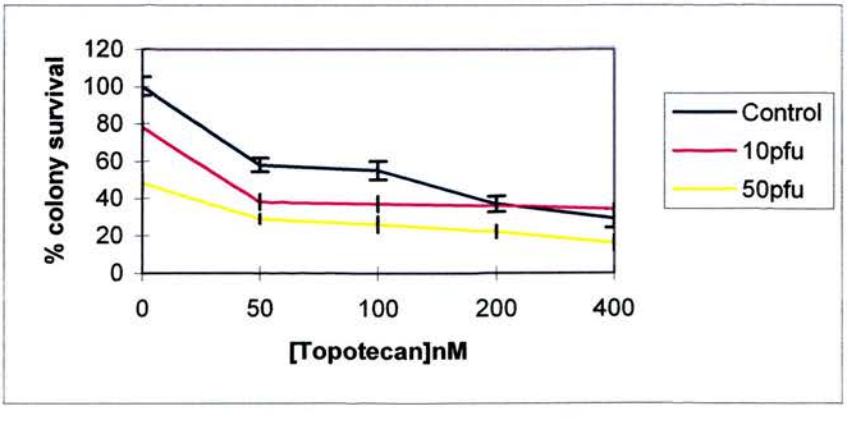


Figure 7.9 – The effect of a combination of topotecan + 24hr exposure to dl1520 in the A2780CP70 cell line

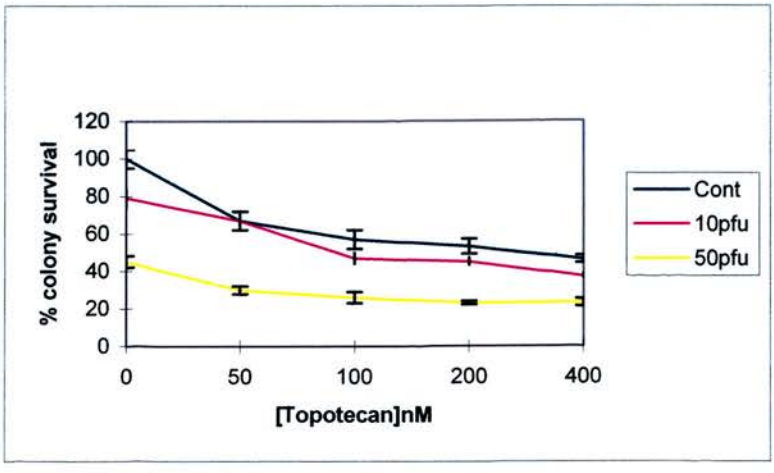


Figure 7.10 – The effect of a combination of topotecan 72hr exposure to dl1520 in the A2780CP70 cell line

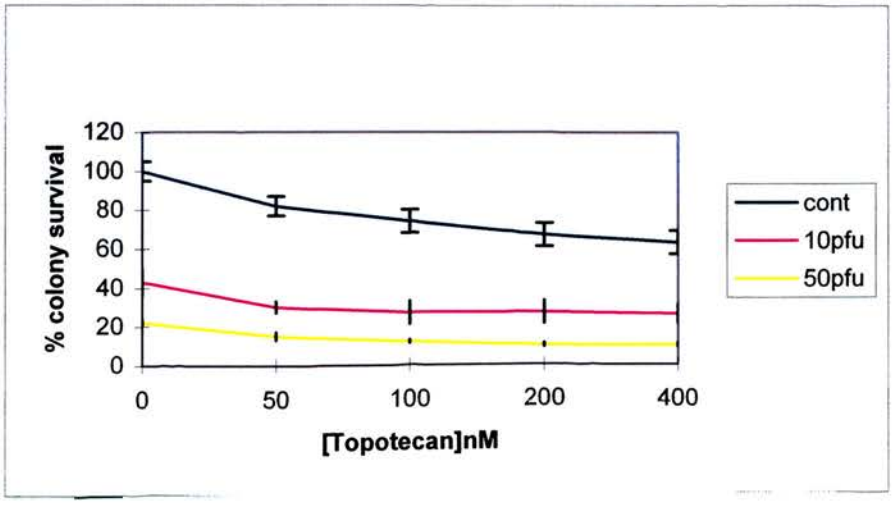


Table 7.5 - Clonogenic survival of A2780 cells following 24 hr exposure to dl1520 and exposure to topotecan as shown. Values expressed as % of control value

[topotecan] uM	Control +/- SEM	10pfu +/- SEM	50pfu +/- SEM
0	100+/-5	77+/-5	70+/-6
50	71+/-5	73+/-6	53+/-5
100	68+/-6	62+/-3	45+/-5
200	67+/-3	49+/-6	43+/-6
400	60+/-1	53+/-6	32+/-6

Table 7.6 - Clonogenic survival of A2780 cells following 72 hr exposure to dl1520 and exposure to topotecan as shown. Values expressed as % of control value

[topotecan] uM	Control +/- SEM	10pfu +/- SEM	50pfu +/- SEM
0	100+/-5	78+/-4	48+/-5
50	58+/-4	38+/-4	29+/-3
100	55+/-5	37+/-4	26+/-4
200	37+/-4	36+/-3	22+/-3
400	29+/-5	34+/-3	16+/-4

Table 7.7 Clonogenic survival of A2780CP70 cells following 24 hr exposure to dl1520 and exposure to topotecan as shown. Values expressed as % of control value

[topotecan] uM	**Control +/- SEM**	**10pfu +/- SEM**	**50pfu +/- SEM**
0	100+/-5	79+/-5	45+/-3
50	67+/-5	67+/-3	30+/-2
100	57+/-5	47+/-3	26+/-3
200	53+/-4	45+/-2	23+/-1
400	46+/-2	37+/-1	23+/-2

Table 7.8 - Clonogenic survival of A2780CP70 cells following 72 hr exposure to dl1520 and exposure to topotecan as shown. Values expressed as % of control value

[topotecan] uM	Control	10pfu	50pfu
0	100+/-5	43+/-7	22+/-2
50	82+/-5	30+/-3	15+/-2
100	74+/-6	27+/-6	12+/-1
200	67+/-6	27+/-6	10+/-1
400	63+/-6	26+/-5	10+/-1

Exposing cell lines matched for p53 function to topotecan and the dl1520 virus, we demonstrate a variable cytopathic effect at different time points. Following 24hr exposure to the virus there is no significant difference between the level of cell kill in A2780 or 2780CP70 cells. After 72 hr virus exposure however there is a significant increase in cell kill in the p53 mutant A2780CP70 cell line. This would be expected, as the A2780CP70 cell line is permissive for viral replication whereas the A2780 cell line, with wild-type p53, should not be. This differential would not be apparent after 24hr virus exposure, as cell death at this point would be expected to be predominantly due to apoptosis. Following viral replication at 72hrs timepoint however the difference in viral replication would be manifest, as a higher cell kill in the A2780CP70 cell line.

Taken together the above experiments indicate that the dl1520 virus can act in an additive fashion with other chemotherapeutic agents at all time points assessed. There was no evidence of antagonism between the agents. After 24 hour exposure to dl1520 there is little difference between the wild-type and p53 null cell lines in terms of their response to the virus. At the later time period of 72 hrs however there was an enormous increase in cell killing in the p53 deficient A2780CP70 cell line. This is probably due to the differential between the p53 wild-type and null being caused by viral replication. At 72 hrs viral replication would be expected to be at a high level whereas at 24 hrs little replication would be evident. As the differential action of dl1520 is due to replication inhibition in p53 wild-type cells this would fit the expected pattern for this virus.

7.5 - Discussion - clonogenic analysis of dl1520 plus cisplatin and topotecan

Cisplatin is a chemotherapeutic drug thought to act by covalently binding to DNA disrupting DNA function and causing cell death in a p53 dependent manner [19, 172].

Topotecan is a topoisomerase I inhibitor that causes cell death in a p53 independent manner [20]. Both of these agents are in common clinical use in oncological practice. We wished to determine if the action of an agent such as dl1520 interacts with such chemotherapeutic compounds in any way, either as an additive or antagonistic way. It is possible that the virus might be used in combination with such agents in a clinical setting.

Combining 24 hrs incubation with dl1520 with a one hour exposure to cisplatin showed similar results in both p53 positive and negative cell lines. In both cases the virus alone caused significant reduction in clonogenic survival and this effect increased with the addition of cisplatin at increasing concentrations. Addition of cisplatin caused an additive effective in both cell lines with no significant difference between the p53 functional or null cell lines. A higher dose of cisplatin was required to give an equivalent level of cell kill in the A2780CP70 as opposed to the A2780 as the p53 null cell line is relatively resistant to this agent.

After 72 hr exposure to the virus however there was a dramatic difference between the two cell lines with a greater cell kill in the p53 null cell line. A similar pattern was found when virus was combined with a 4hr exposure to topotecan with no significant difference between the two cell lines at 24hrs virus exposure. The longer exposure in the A2780CP70 cell line led to a significant reduction in cell survival both with and without the addition of chemotherapeutic agents.

The most obvious explanation for this is a difference in viral replication. After 24hrs exposure the dl1520 virus would be expected to inoculate both p53 functional and mutant cell lines. It is accepted that the virus can trigger cell death by apoptosis in both p53 positive and negative cell lines [136] and that this effect can be greater in p53 competent cell lines. This effect is due to a p53 mediated action triggered by the adenoviral E1A protein following viral inoculation into cells and is a protective

mechanism designed to prevent viral replication causing widespread tissue damage [119]. This mechanism of apoptotic cell death could occur in an additive fashion with both the chemotherapeutic agents tested and would be expected to occur soon after viral inoculation in susceptible cells.

After 72hrs virus incubation however the effect of viral replication would be more important. Adenovirus causes most of its cytopathic effect during the late phase of the viral replication cycle after 48 hrs [79]. Following this cytopathic effect virus is shed and viral integration into neighbouring cells can occur. Where replication of virus is prevalent therefore a greater cytopathic effect is seen. This occurs following the longer viral infection in the p53 deficient cell line. In the p53 competent cell line however little viral replication would be expected as in theory the virus would trigger p53-mediated apoptosis before replication could take place. This would abrogate viral replication diminishing the cytopathic effect and also reducing viral spill into the culture medium releasing less virus for subsequent inoculation of cells. In the p53 null cell line this apoptosis would be significantly reduced, meaning viral replication and ultimately increased cytolysis could happen.

These results confirm the p53 selectivity of viral replication with increased cell death in p53 null cells following a 72hr viral incubation. It is clear however that the virus also causes increased cell death in p53 competent tumour cells when combined with chemotherapy and one can hypothesise that this effect is mediated by apoptotic cell death. Initial cell death is therefore to be expected in p53 competent cell lines in the early stages following viral inoculation but there should not be a large increase in this effect following a longer inoculation. One weakness of the above study is that no investigations of the mechanism of cell death were performed. This could be an area of future research as the nature of cell death in these two cell lines is of significant interest.

The virus has been shown previously to act in an additive fashion to decrease tumour

size with standard chemotherapeutic agents in a murine xenograft model (cisplatin and 5-FU) when administered intra-tumourally or intravenously [1]. Other workers have also demonstrated an additive effect with dl1520 and chemotherapy in a tissue culture model using primary lung cancer cell lines [7]. Our results again demonstrate that the dl1520 adenovirus can act in an additive fashion with other commonly used chemotherapeutic agents. We demonstrate little additive benefit of cisplatin when used with A2780CP70, the p53 deficient cell line. Using either 10pfu or 50 pfu of virus demonstrates high levels of cell killing even without the addition of cisplatin. To demonstrate synergy in this setting it may have been beneficial to use lower doses of virus. It is also important to bear in mind that most head and neck SCC samples show heterogenicity of p53 expression, in contrast to the cell lines investigated here [173] [174]. In a clinical setting therefore any treatment regime would probably have to target both p53 wild-type and mutant cells.

It is important that dl1520 shows no evidence of antagonism to standard chemotherapy agents. In the clinical setting it is likely that a viral therapy such as dl1520 would be used in combination with other treatment modalities such as chemotherapy This was the case in the recent successful phase II trial of dl1520 in combination with chemotherapy in head and neck cancer [139].

It is logical to combine a gene therapy treatment that is selective for p53 negative tissue with another treatment modality that can target tumour cells with functional p53. This can give a combined therapy modality that can destroy cancer cells with both functional and non-functional p53. It is well recognised that the loss of p53 function contributes to chemo- and radio- resistance in many tumours which initially respond to these therapies but become refractory to treatment [19]. A treatment that specifically targets such resistant tumour cell lines and can be used alongside conventional treatments, which target p53 functional cells, is therefore desirable. The above experiments indicate that dl1520 could be used in such a manner. There is a potential area of concern in that the

dl1520 appears to act in an additive fashion with both p53 functional and dysfunctional cell lines. In theory this could increase the risk of damage to non-tumour tissues in clinical trials, especially if systemic delivery was used. To date however, no such clinical effect has been noted[92].

7.6 - Summary

These in-vitro studies therefore demonstrate the following:

1. Dl1520 can act to kill cancer cells with both functional and dysfunctional p53 in an additive fashion when combined with chemotherapy.

2. The effect of dl1520 differs with the p53 status of cells in a time dependent fashion. For the differential effect of the virus between p53 wild-type or mutant cells to manifest, a sufficient time interval must elapse to allow for viral replication. The difference in effect is probably due to differential viral replication, which is maximal at about 72 hrs following inoculation.

CHAPTER EIGHT – A SINGLE INJECTION OF dl1520 INTO MURINE XENOGRAFTS

8.1 - Materials and methods

8.1.1 - Hexon stain for adenoviral protein expression

Paraffin sections were derived from tumour xenografts, grown as detailed below. The tumours were preserved in formalin and processed in a standard fashion prior to wax embedding and sectioning. The sections were then dewaxed in three changes of xylene for 5 minutes each and rehydrated through a series of 100%, 90% and 70% ethanol and distilled water. Immersing the slides in 3% hydrogen peroxide in methanol for 45 minutes quenched endogenous peroxidase activity and the slides were rinsed in two changes of PBS. The sections were then treated with protease (Pronase, Meridian diagnostics 1.7mg/ ml in PBS) for 20 minutes in a humidified chamber at 35° C and the slides were rinsed in two changes of PBS. Casein solution was used to block non-specific (Power Block casein solution, Biogenex diluted 1:10 in PBS).

The mouse primary anti-adenovirus monoclonal antibody Ig1k isotype (Chemicon) was used at a dilution of 1:1000 and 100 µl was added to the section and a coverslip added. This was incubated for 45 mins in a humidified chamber at 35°C and slides were rinsed in two changes of PBS.

Subsequent antibodies were provided from the Biogenex Super Sensitive Immunodetection System, Biogenex. 100 µl of a biotinylated secondary antibody was added to each section and incubated for 20 mins at room temperature and slides were rinsed in two changes of PBS. Streptavidin-horseradish peroxidase conjugate (100 µl) was added to each section and incubated for 20 mins at room temperature and slides were rinsed in two changes of PBS. DAB was used as a chromagen with 100 µl added to each section for 8 mins. Slides were then rinsed in distilled water, counter-stained in Mayer's hematoxylin for 2 mins and dipped in Ammonia water for 10 seconds. Sections were dehydrated through 70%, 90% and 100% alcohol prior to being immersed in three changes of xylene. Sections were mounted using permount. Murine xenograft sections

known to harbour adenovirus were used as positive controls and pbs injected samples as negative controls. All analyses were performed three times. Counts of cell infectivity were made in a minimum of five high-powered fields for each section.

8.1.2 - Protocol for growth of murine xenografts

8.1.2.1 - Animals

Six week old athymic MFU nude mice were supplied and maintained according to standard protocols.

8.1.2.2 - Raising of xenografts

Cell lines A2780 and A2780CP70 were cultured as described previously. Cells were trypsinised and counted and centrifuged at 1500 rpm for 5 minutes to form a cell pellet. The pellet was resuspended in PBS. 10^7 cells in 100ul of PBS were injected subcutaneously at two sites per mouse. Each experiment was performed with two cohorts of subjects.

8.1.3 - Preparation of dl1520 adenovirus

Dl1520 is formulated as sterile viral solutions in TRIS buffer (10 mM TRIS pH 7.4, 1 mM $MgCl_2$, 150 mM NaCl, 10% glycerol). The product is supplied frozen in single-use vials and contains no preservative. Virus was thawed in a biological safety cabinet and diluted in PBS to give a concentration of 10^8 pfu in 100µl of solution.

8.1.4 - A single intra-tumoural injection of dl1520 in A2780 and A2780CP70

8.1.4.1 - Injection of dl1520 adenovirus

Once palpable tumours were established, at about day five, tumours were injected with either:

1. 100ul PBS.

2. 10^8 plaque forming units (pfu) dl1520 in 100ul PBS.

Injections were made by inserting a 23-gauge needle into each quadrant of the tumour and infiltrating virus along the tract formed as the needle was withdrawn. Four needle tracts were used for each tumour and 30 tumours were grown for each group. Tumours were harvested at 24hrs, 72 hrs or 14 days determined by the maximal accepted size of the xenografts. Tumours were fixed in 4% phosphate buffered formalin.

8.2 - Results

The tumours were harvested at three timepoints following virus injection – 24hrs, 72hrs and 14 days. These three time-points were chosen to allow for the effects of viral replication, which would be expected to be maximal at 72hrs and to tail off after this time. Following tumour harvest and processing, histological specimens from the xenografts were assessed for the presence of adenoviral hexon protein using immunohistochemistry. A comparison was then made of the capacity of the virus to exist and replicate in tumours identical except for their p53 status.

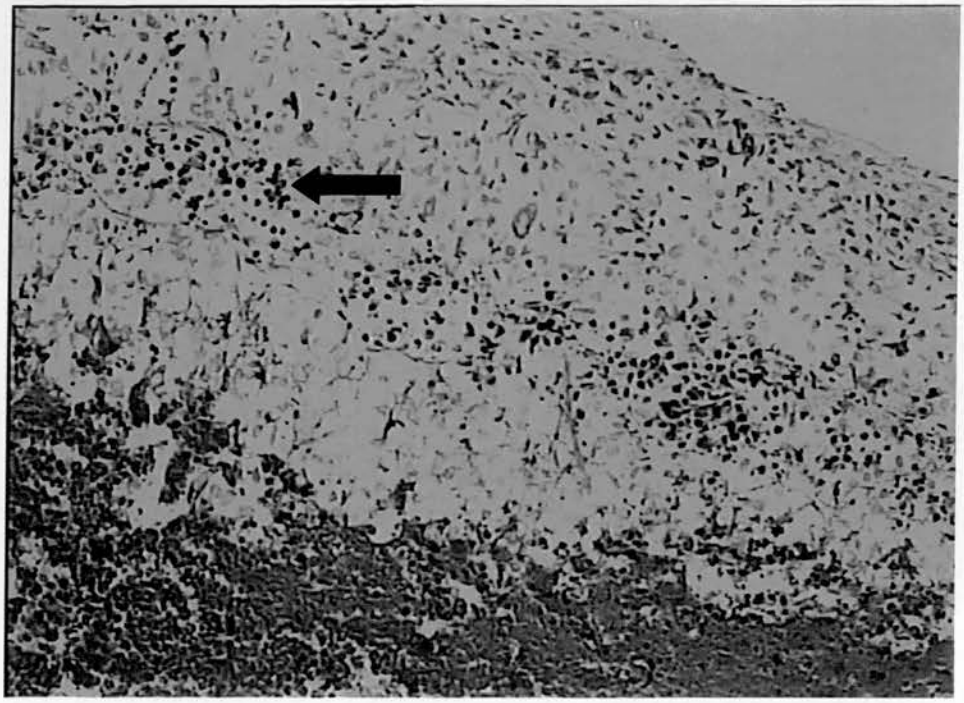
It became apparent during analysis that the virus tends to collect in cells located at the interface between the necrotic tumour centre and the interstitium as has been previously demonstrated (Fig 4.1). The amount of cells in which virus was detected at this interface area was therefore used to assess viral presence in this experimental model. Assessments were made of:

1. The % of cells staining positive for adenoviral hexon protein within each high powered field at the interface area. This value gave an estimate of the density of virally infected cells.

2. The % of high-powered fields at the interface containing any cells staining positive for adenoviral hexon protein. This value gave an estimate of the distribution of virally

infected cells in the area permissive for viral replication.

Figure 8.1 – positive staining for adenoviral hexon protein. Black arrow point to positive (dark brown) cells



The % of cells staining positive for adenoviral hexon protein at the interface between necrotic tumour centre and tumour interstitium was assessed. This assessment was made by looking at each high-powered field that contained any cells staining positive for adenovirus. The % of positive cells out of each high-powered field was calculated after an assessment of at least four fields per histology slide, with values expressed +/- SEM.

This gave values of 18+/-4.9 % for A2780CP70 and 0 % for A2780 at 24hrs; 51.2+/-8.5 % for A2780CP70 and 3.3+/-0.7 %for A2780 at 72hrs and 32.3+/-10.8 % for A2780CP70 and 2+/-0.6 % for A2780 at day 14. These differences were significant $p<0.05$ at 24hrs; p at <0.05 at 72hrs and $p<0.05$ at day 14 when analyzed with Student's t-test (see fig 8.2).

The % of high-powered fields containing any cells staining positive for adenoviral hexon protein at the interface between necrotic tumour centre and tumour interstitium was assessed. The total number of high-powered fields surrounding the necrotic centre of each tumour was counted and then the % of these containing cells staining positive for adenovirus was calculated.

This gave values of 20+/-1 % for A2780CP70 and 0 % for A2780 at 24hrs; 56.7+/-6.2% for A2780CP70 and 16.7+/-6.8 % for A2780 at 72hrs and 25.6+/-6.3 % for A2780CP70 and 10.5+/-3.7 % for A2780 at day 14. These differences were significant with $p<0.05$ at 24hrs; $p<0.05$ at 72hrs and $p<0.05$ at day 14 (see fig 8.3).

Figure 8.2 – The % of cells within each high-powered field where virus was detected staining positive for adenoviral hexon protein

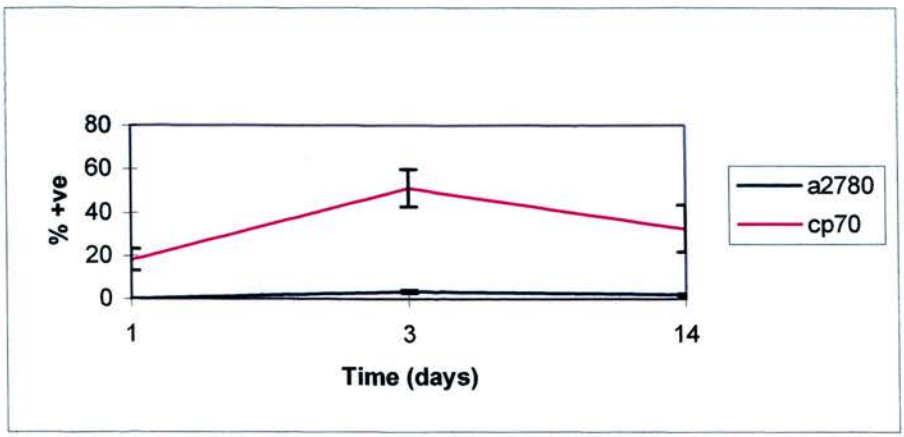
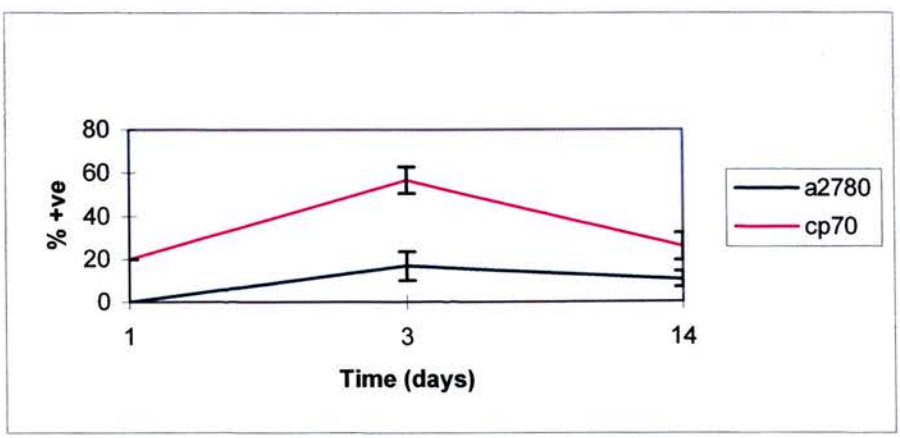


Figure 8.3 – The % of high-powered fields with cells staining positive for adenoviral hexon protein



The size of tumours was monitored over the 14 day cycle following viral injection. No difference in tumour size was found between the two cell lines. This was to be expected given that a single dose of virus was administered of a relatively low dose.

These results indicate that the dl1520 virus can replicate in both p53 null and competent cell line xenografts in a murine model as in both cell types the level of virus rose between 24 and 72 hrs. The level of viral presence and replication is significantly higher in p53 negative cell lines at all time points assessed. This difference is significant at all time points and applies both in terms of viral density and viral distribution within the xenografts. This supports the hypothesis that the dl1520 virus can replicate preferentially in p53 deficient cells in a tumour model. The difference is present at all time-points assessed but is maximal at 72 hrs. This would suggest that the difference is due to a differential level of viral replication and not simply viral survival as there was an increase in viral presence at 72 hrs compared to 24 hrs.

8.3 - Discussion

8.3.1 - Replication and Spread in a Matched p53 Tumour Model

Using p53 matched human tumour xenografts in a murine model demonstrated that the dl1520 virus replicates and spreads preferentially in p53 negative tumours. Viral presence was assessed in two ways:

1. As a percentage of the interface between the necrotic tumour centre and the surrounding cells where virus was detected.

2. As a percentage of cells where virus was localised in those areas where virus was detected.

These results demonstrate that in both cell lines there is maximal virus detected at 72hrs,

rising from the 24-hr time-point and then declining at the 14 day time-point. This is in keeping with the expected pattern of virus replication within the tumour xenografts in both cell lines which would be maximal at 72 hrs, and would be greater in p53 null cell lines. In the A2780CP70 p53 null cell lines there was evidence of more widespread detection of virus where hexon expression was counted as a percentage of fields where virus was detected and that within these fields a higher percentage of virus was detected. It is reasonable to presume a higher level of virus replication therefore in the A2780 CP70 cell line.

Previous work has demonstrated a higher level of tumour growth inhibition in p53 null cell lines using the dl1520 virus [1]. This was presumed to be an effect of differential viral replication but no measurement of virus within the xenografts was performed. We demonstrated that there is a differential level of virus within the p53 matched tumour cell line xenografts and that this is due to increased replication of the virus in the p53 mutant cell line.

These experiments demonstrate that the virus replicates preferentially in p53 deficient cell lines in a xenograft model. In this experiment infectivity was not 100%. It is known the dl1520 is less infective than the wild type adenovirus, which may explain this feature, which is in keeping with similar previous experiments [5]. It is clear that the virus can also exist and replicate in tumour cells with a functional p53 but to a far lesser extent. A small number of groups have reported results with the dl1520 virus disputing the p53 selectivity of cell killing. Rothmann et al conducted tissue culture experiments with various human cell lines of both functional and dysfunctional p53 status [127]. They reported that replication of the virus was independent of p53 status in many cell types. Our results, using a xenograft model, would indicate that if exactly matched cell line is used the level of replication, and therefore any likely anti-tumour activity, is related to p53 status of the tissue.

CHAPTER 9 - EFFECT OF VARYING THE CARRIER MEDIUM ON VIRAL DISTRIBUTION

9 - Comparison of a single intra-tumoural injection of dl1520 in a2780cp70 tumour xenografts using varying carrier media

9.1 – Materials and methods

Tumour xenografts of A2780CP70 were raised as described earlier. Once tumours were established they were injected with the investigational material. Four cohorts of 30 tumours each were injected with either:

1) 10^8 plaque forming units (pfu) dl1520 in 100ul PBS.
2) 10^8 pfu dl1520 in 100ul 1% lignocaine.
3) 10^8 pfu dl1520 in 100ul 200iu/ml hyaluronidase solution.
4) 10^8 pfu dl1520 in 100ul 1% lignocaine containing 200iu/ml hyaluronidase.

Tumours were harvested at 4hrs, 24hrs, 72 hrs or at a period up to 14 days determined by the maximal accepted size of the xenografts. They were then fixed in 4% phosphate buffered formalin.

An experiment to determine if varying the carrier medium could enhance the distribution of dl1520 adenovirus was devised. Xenografts of the A2780CP70 p53 deficient cell line were assessed for the presence of adenoviral hexon following harvesting at different time-points following a single injection of dl1520 in various carrier media. Distribution was calculated as a % of high-powered fields for each histological section containing any cells staining positive for adenoviral hexon protein. As in the previous experiment, the necrotic centers of tumours were not included in this assessment, as it is known the virus exists outwith this area.

An injection of the dl1520 adenovirus was made in to tumour xenografts as detailed in materials and methods. The time-points used for assessment were with tumour harvest at

4 hrs; 24 hrs; 72 hrs and 14 days following a single injection. The carrier media used were standard PBS; 1% lignocaine; 200iu/ ml hyaluronidase and 1% lignocaine with 200iu/ ml hyaluronidase. Following tumour harvest the specimens were preserved in formalin and processed for immunohistochemical staining with the hexon anti-body as detailed previously. Distribution of virus was assessed by calculating the % of high-powered fields with any cells staining positive for virus in each histological section (see figures 9.1-9.4).

9.2 - Results

Values are shown along with the standard deviation for each value. Student's t-test was applied to these results to determine their statistical significance (as shown by the p values in parentheses).

At **four hours** following the injection of dl1520 the % of fields with cells +ve positive for hexon protein was:

1. 12.5+/-3.5 % with PBS as a carrier medium
2. 20.2+/-2.2 % with 1% lignocaine as a carrier medium ($p<0.05$)
3. 19.1+/-2.4 % with 200iu/ml of hyaluronidase as a carrier medium ($p<0.05$)
4. 31.9+/-5 % with 200iu/ml of hyaluronidase in 1% lignocaine as a carrier medium ($p<0.01$)

There is a significant increase in distribution in groups 2-4 compared with group 1 (control) with the p values as shown above (see fig 5.1). This indicates that at the four-hour time-point the effect of lignocaine and hyaluronidase is to increase the distribution of the adenovirus within tumour xenografts following a single intra-tumoural injection.

At **24 hours** following the injection of dl1520 the % of fields with cells +ve positive for hexon protein was:

1. 28.5+/-3.8 % with PBS as a carrier medium.
2. 62+/-6.2% with 1% lignocaine as a carrier medium ($p<0.001$).
3. 57+/-6.4% with 200iu/ml of hyaluronidase as a carrier medium ($p<0.001$).
4. 63.5+/-4.9 % with 200iu/ml of hyaluronidase in 1% lignocaine as a carrier medium ($p<0.0001$).

There was a significant increase in distribution in groups 2-4 compared with group 1 with the p values as shown above when Student's t-test was applied (see fig 5.2). This indicates that at the twenty-four hour time-point the effect of lignocaine and hyaluronidase is to increase the distribution of the adenovirus within tumour xenografts following a single intra-tumoural injection.

At **72 hours** following the injection of dl1520 the % of fields with cells +ve positive for hexon protein was:

1. 44.2+/-5.2 % with PBS as a carrier medium
2. 71.8+/-5.6 % with 1% lignocaine as a carrier medium ($p<0.001$)
3. 78.5+/-4.2 % with 200iu/ml of hyaluronidase as a carrier medium ($p<0.0001$)
4. 64.6+/-9.4 % with 200iu/ml of hyaluronidase in 1% lignocaine as a carrier medium ($p<0.05$)

There was a significant increase in distribution in groups 2-4 compared with group 1 with the p values as shown above when Student's t-test was applied (see fig 5.3). This indicates that at the seventy-two hour time-point the effect of lignocaine and hyaluronidase is to increase the distribution of the adenovirus within tumour xenografts following a single intra-tumoural injection.

At **14 days** following the injection of dl1520 the % of fields with cells +ve positive for

hexon protein was:

1. 28.7+/-9.1 % with PBS as a carrier medium
2. 50.9+/-6.4 % with 1% lignocaine as a carrier medium ($p<0.05$)
3. 56.1+/-5.7 % with 200iu/ml of hyaluronidase as a carrier medium ($p<0.01$)
4. 49.4+/-8.5 % with 200iu/ml of hyaluronidase in 1% lignocaine as a carrier medium ($p<0.05$)

There was a significant increase in distribution in groups 2-4 compared with group 1 with the p values as shown above when Student's t-test was applied (see fig 5.4). This indicates that at the fourteen-day time-point the effect of lignocaine and hyaluronidase is to increase the distribution of the adenovirus within tumour xenografts following a single intra-tumoural injection.

Figure 9.1 – typical viral distribution at 72hrs using PBS as a carrier

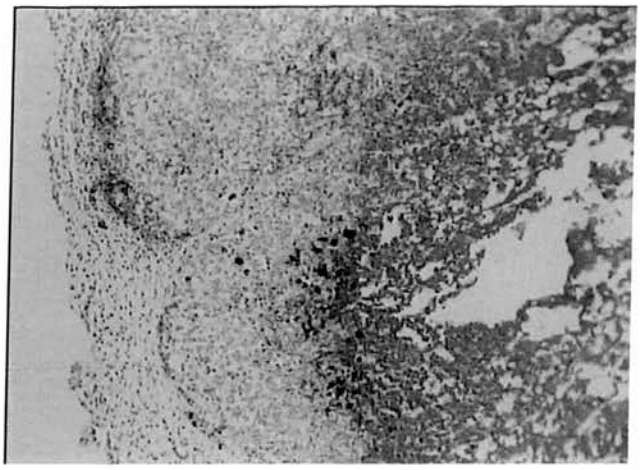


Fig 9.2 – typical viral distribution at 72hrs using lignocaine as a carrier

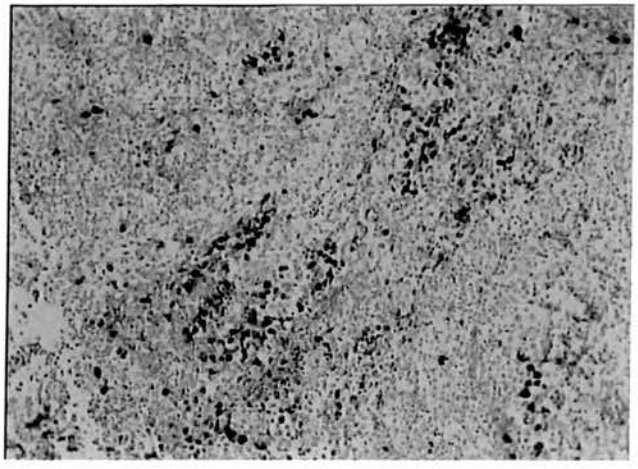


Fig 9.3 – typical viral distribution at 72hrs using hyaluronidase as a carrier

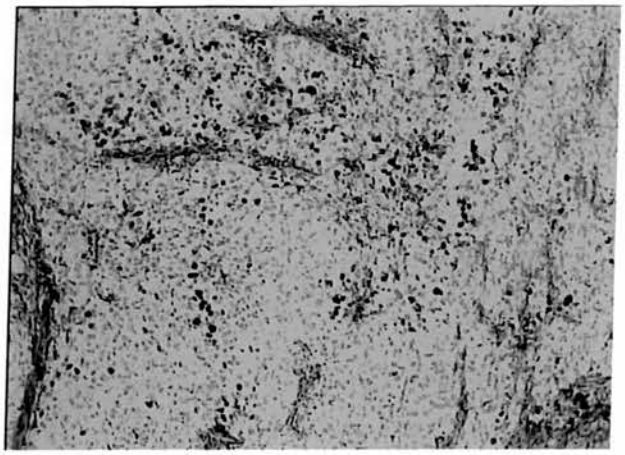


Fig 9.4. – typical viral distribution at 72hrs using hyaluronidase and lignocaine as a carrier

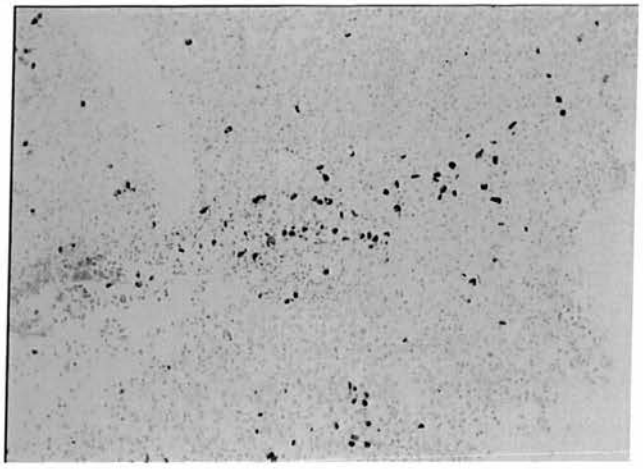
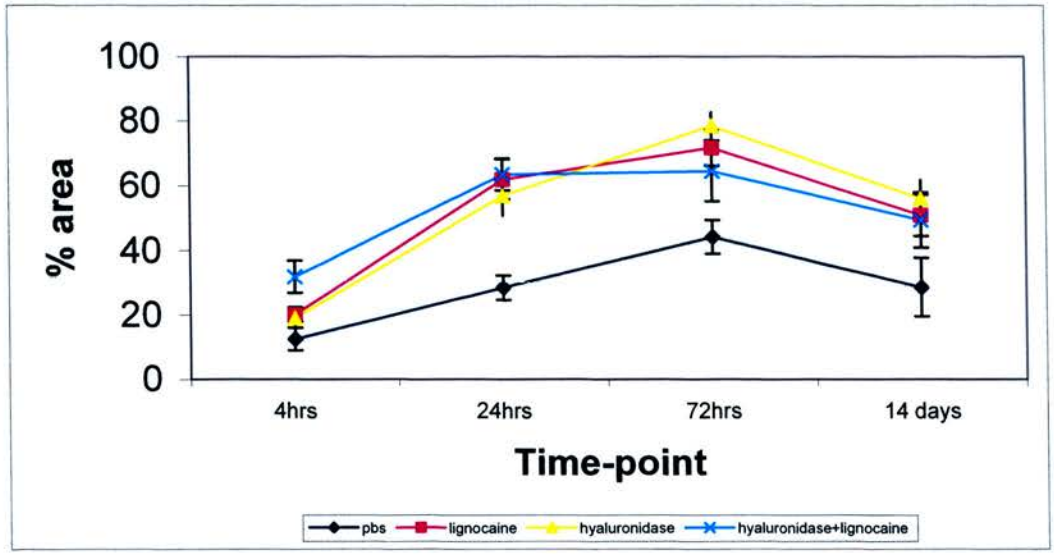


Figure 9.5 - Distribution of dl1520 virus following a single intra-tumoural injection using various carrier media demonstrated by positive staining for adenoviral hexon protein



These results demonstrate a significant increase in the distribution of dl1520 adenovirus when either hyaluronidase or lignocaine is used as a carrier medium to deliver the virus. Combining lignocaine and hyaluronidase as a carrier medium did not significantly increase this advantage compared to using either agent singly. This difference was apparent at all time points assessed indicating that the effect is due at least in part to an immediate distribution effect. The different carrier media do not adversely affect the survival of the virus as indicated by the fact that virus was detected up to 14 days post injection.

This shows that a better distribution of the adenovirus dl1520 throughout a tumour can be achieved by varying the carrier medium. The agents used are in common clinical use and as such are known to be non-toxic in the required dose range. It is possible that

increasing the distribution of a virus in this way could increase its therapeutic effect.

9.3 - Discussion

Using a murine xenograft model it was demonstrated that by varying the carrier medium used to deliver the dl1520 adenovirus the distribution of the virus could be increased. Both lignocaine and hyaluronidase has this effect when compared to using PBS as a carrier medium. This effect is noted using each agent alone or in combination.

Hyaluronidase is an enzyme that depolymerises hyaluronic acid in the ground substance of tissue interstitium. Hyaluronic acid is a mucopolysaccharide present in the ground substance of tissues and forms highly viscous fluids. Once depolymerised it forms a watery fluid, which facilitates distribution of substances within the ground substance of tissues. It has long been known to increase the spread of local anaesthetic when utilised as a subcutaneous or intra-dermal injection and can decrease the time taken for distribution [175]. An increase in the area of distribution has also been noted previously when hyaluronidase is combined with local anaesthetics [145]. One can speculate that these effects might both be noted when injecting adenovirus intra-tumourally hence explaining the increased viral distribution, although this mechanism of action has not been confirmed.

Lignocaine is a commonly used local anaesthetic agent. It acts by blocking the voltage dependent sodium channels in excitable tissues thereby preventing impulses travelling in pain sensitive nerves [176]. This blocking is not however confined to nervous tissue and the smooth muscle of arterioles is also affected causing a relaxation of these cells with resultant vasodilatation [177, 178]. Local vasodilatation of arterioles would increase the blood flow around the tumour xenograft where the intra-tumoural injection was made, potentially increasing the spread of a substance within the tumour. This increased blood flow might explain the observed increase in viral distribution, although this cannot be

confirmed.

As the hyaluronidase and lignocaine act by independent means to increase the distribution of virus it is possible that they could have an additive effect although no synergy was demonstrated in this series. Further studies might be able to explain this lack of synergistic effect. These results have considerable significance for viral therapy in general. It has been noted previously that dl1520 tends to be seen preferentially around the interface between the necrotic central areas of tumours and the peripheral tumour cells [1, 137]. Any process whereby virus can be encouraged to spread within the tumours could lead to an enhanced anti-tumour activity as a greater proportion of the tumour could be affected at any one time. It has previously been demonstrated in a xenograft model that increasing viral distribution by other means, namely using multiple divided doses for administration and increasing the volume of viral suspension, could lead to enhanced anti-tumour effect in p53 null cell lines [131, 132]. It would follow that increasing distribution by altering the carrier medium could have the same effect. This effect could benefit any gene therapy protocols where an adenovirus is used as a carrier and a local injection is utilised.

There are safety issues concerned with any therapy that might increase dissemination of tumour cells following an intra-tumoural injection. As lignocaine is frequently used in all surgical fields with no adverse effects it is reasonable to assume that the local vasodilatation it causes does not lead to such dissemination. The safety of intra-tumoural injection of hyaluronidase would have to be further investigated prior to any clinical trials being undertaken. In theory it is possible that the action of hyaluronidase in disrupting the ground substance of tissues could lead to a risk of embolisation and possible metastasis of tumour deposits. Further work using an animal model to look at this aspect of treatment would be required before any trials in humans would be suggested.

9.4 Summary of results

The above laboratory work has shown the following points in a tissue culture and murine xenograft model.

1. The virus can act in an additive fashion with standard chemotherapeutic agents to kill cancer cells. Cells of both mutant and wild type p53 status can be killed but the effect is very much greater in p53 null cells if sufficient time for viral replication is allowed.

2. Following a direct intra-tumoural injection spread and replication of the virus is influenced by the p53 status of the cell line used. Both spread and replication of virus are greatest in p53 null cell line tumours.

3. Varying the carrier medium can influence the spread of the virus with no detrimental influence on viral replication. Two commonly used agents, lignocaine and hyaluronidase, were used to successfully increase the spread of the virus following a single intra-tumoural injection.

We have therefore shown several points that would support the use of dl1520 as a selective anti-cancer agent. These results support the concept that this selectivity is based on the p53 status of tumour cells.

CHAPTER 10 - CONCLUSIONS AND SUGGESTIONS FOR FURTHER RESEARCH

10 - Conclusions

This thesis is concerned with both laboratory based and clinical studies concerned with the selectivity, delivery and mechanism of action of the novel anti-cancer agent dl1520. This adenovirus represents part of an exiting new field of potential treatments for cancer, that of gene therapy. The virus acts by exploiting a fundamental genetic defect that is present in most human solid malignancies, mutation of the p53 tumour suppressor gene.

The above results of the series of laboratory experiments demonstrated success in adding to our knowledge of the mechanism of action and p53 selectivity of the virus; of how to optimise virus delivery and whether the agent can be used in combination with chemotherapy. The results of a clinical trial involving the dl1520 attenuated adenovirus have added to our knowledge about the likely mechanism of action of the agent and about its safety in clinical use.

The aims of the thesis were set out as follows:

Laboratory experiments were performed to further clarify:

1. **The mechanism of action of dl1520**
2. **The p53 specificity of the virus both in vitro and in vivo**
3. **Optimisation of intra-tumoural virus delivery**
4. **The effect of combining the virus with other standard chemotherapeutic agents**

The clinical trial was devised to evaluate in a human model:

1. **The mechanism of action of dl1520**
2. **The p53 specificity of the virus in vivo**

3. **Optimisation of intra-tumoural viral delivery**
4. **Tolerance of administration of dl1520 in a select patient group**
5. **Immune cell response to the virus**
6. **Level of tumour necrosis following viral administration**

10.1 - Laboratory based experiments

10.1.1 - The mechanism of action of dl1520

Significant information as to the mechanism of action of the agent was gleaned from a clonogenic analysis of the effect of using the virus in conjunction with chemotherapeutic agents (cisplatin and 5-fluouracil). It was found that the virus had a significantly enhanced level of cell kill when used against a p53 null cell line as opposed to the p53 functional equivalent line (A2780CP70 compared to A2780). This difference only manifests after the virus is left for a 72 hr incubation with the cells as opposed to a 24 hr incubation, where levels of cell kill are roughly comparable between the cell lines. We propose that this time dependent difference shows that the mechanism of cell killing following inoculation with the virus is cytolysis following viral replication. It requires a 72hr inoculation with the virus to allow time for viral replication to become maximal to show the increased cell kill in the p53 null cell line which is permissive for replication of the dl1520 virus. After 24 hrs there is no difference between the two, as viral replication has not progressed sufficiently to demonstrate a replication dependent difference. This experiment therefore supports the hypothesis that the dl1520 virus kills cells mainly by cytolysis, and this is the mechanism which allows selectivity between tissue of wild-type or mutant p53.

10.1.2 - The p53 specificity of the virus both in-vitro and in-vivo

The same experiment also gives information to support the theory of p53 selectivity of the virus. As detailed above a significant increase in cell kill following inoculation with

the virus was demonstrated in p53 null cell line as compared to the similar cell line with a functional p53. This difference was time dependent and only manifest after a 72 hr time period, as would be predicted where the differential effect was dependent on viral replication. This replication in an adenovirus is maximal after 72 hrs.

A tumour xenograft model was also used to investigate any differences in viral replication in tumours grown from cell lines similar except for p53 status. A single injection of the virus was made into tumours of differing cell type and the tumours harvested at different time points following the injection. It was shown that both viral distribution and quantity was significantly higher in p53 mutant xenografts as compared to tumours grown from p53 competent tissues. This experiment successfully demonstrates p53 selectivity of dl1520 in a tumour xenograft in-vivo model.

10.1.3 - Optimisation of intra-tumoural virus delivery

Using a tumour xenograft model it was successfully demonstrated that delivery of the virus could be increased by varying the carrier medium used for injection. Two agents were tested:

1. Lignocaine - a commonly used local anaesthetic agent which can also act as a vaso-dilator.

2. Hyaluronidase – an enzyme, which acts to liquefy tissue interstitium, leading to increased distribution of injected agents.

Both of these agents caused an increased distribution of the virus when used as a carrier medium. The activity of the virus might be increased with optimisation of its distribution and it is possible that this could lead to increased activity of the virus.

Several further experiments to clarify this could be undertaken. The same xenograft model could be used with a higher dose of virus or with multiple doses to deliver sufficient virus to cause tumour shrinkage. Tumours grown on the same mice could have either standard PBS as a carrier or either hyaluronidase or lignocaine. A comparison of effect on tumour growth could then be made. It would also be useful to investigate whether the use of these agents in direct intra-tumoural injection leads to an increased risk of metastases and a murine xenograft model could be used to investigate this.

If these studies were promising a clinical trial could be considered using either agent to administer the virus. It would however have to be clear that neither agent was likely to cause seeding of tumour cells to distant sites.

10.1.4 - The effect of combining the virus with other agents

A model of anti-tumour activity of the virus using clonogenic analysis was devised to allow investigation of the effect of combining the virus with either cisplatin or 5-fluorouracil. In this model it was successfully shown that the virus can act in an additive fashion with chemotherapeutic agents. This effect is seen at both 24hrs and 72 hrs incubation with the virus and with both p53 wild-type and mutant cell lines. From this we can deduce that the virus could be used alongside such agents in the treatment of cancer, particularly in those cases where chemotherapy alone might have been used. There was no evidence of antagonistic action between the virus and other agents.

The same model could be used in the future to investigate the effect of using the virus with other chemotherapeutic agents. It would also be possible to test the agent in combination with chemotherapy in an in-vivo model, using a murine xenograft model. The virus could be delivered into tumours by direct intra-tumoural injection along with systemic administration of whichever chemotherapy agent is under investigation. Such

studies have already been successfully undertaken using both cisplatin and 5-fluorouracil and this model could be similarly used to test other agents [1].

10.2 - Clinical trial of a single injection of dl1520

10.2.1 - The mechanism of action of dl1520

It had been hoped that the clinical trial of a single intra-tumoural injection of dl1520 would add to our knowledge of the mechanism of action of the virus in-vivo following direct intra-tumoural administration. It was found however that following a single injection we could not grossly demonstrate significant tumour necrosis. As mentioned in the discussion section this was probably due to the relatively small amount of virus being used. A small amount of cytolysis was however noted around the areas where adenovirus was detected. This supports the theory that the virus can kill cancer cells by cytolysis. This effect was only noted in specimens harbouring a mutant p53 genotype. By contrast, in normal tissues injected with the virus a relatively high level of apoptosis was noted, significant at th 24 hr time-point. This seems to demonstrate that normal tissues respond to the virus by allowing apoptotic cell death, which would be expected to be an early event. These findings give further evidence that the virus can kill p53 mutant cancer cells by cytolysis but in normal, p53 wild-type tissues cause apoptosis as an early event. This is in keeping with the purported mechanism of selectivity of the agent, which should in theory give this effect in p53 competent cells. This effect was not seen in p53 wild-type tumour cells perhaps indicating a defect elsewhere in the p53 mediated apoptosis pathway.

10.2.2 - The p53 specificity of the virus in-vivo

The above clinical trial demonstrated convincingly that the virus exists preferentially in tumour tissue rather than normal tissue. The only two samples of normal tissue where

the virus was detected had abnormalities of p53 on immunohistochemical staining. It could be assumed that this difference between normal and tumour tissue was due to the likely differing p53 status of the tissues. Gene sequencing was not however performed on the normal tissue biopsies and so it is only possible to make deductions from the immunohistochemistry results. We were not able to demonstrate a statistically significant difference in viral presence or replication between tumour samples of differing p53 status. More virus was found in p53 mutant tumours, but as only 3 tumours had a wild-tpe p53 sequence, a statistical significance could not be demonstrated.

The most we can confidently say from this trial is that the virus preferentially survives and replicated in tumour tissue as opposed to normal tissue following direct injection. This is likely to be related to p53 status, but we were unable to demonstrate this in a statistically significant manner. Further studies to pursue this might include similar studies of a larger size where more tumours of wild-type p53 status are investigated. Analysing normal tissue samples by gene sequencing might also be of benefit if a similar trial were to be conducted in the future. We could then say with certainty whether these normal tissue samples did have a normal p53 or if the gene for this protein had been damaged as part of a field change. Our study demonstrated that the dl1520 virus can exist in normal tissue, in this case in two samples out of fifteen, but it is not clear if these samples all had a fully functional p53 protein. Both the samples where virus was found did demonstrate positive immunohistochemical staining for p53, possibly indicating abnormal protein.

10.2.3 - Optimisation of intra-tumoural viral delivery

The clinical trial showed that direct intra-tumoural delivery of the virus is possible and safe. The patients tolerated injections and no significant side effects were noted. It has been previously noted that multiple rather than single injections lead to optimisation of delivery and spread of the virus. An in-vivo animal model has also shown that

maximising the volume of diluent used to administer the virus can lead to increased viral spread [132]. It is therefore likely that if the trial was repeated using multiple injections rather than a single injection, wider distribution of the virus would have been found. Other trials have demonstrated that higher doses of this virus can be safely used in similar groups of patients and so multiple injections of the same dose (10^{10} pfu) could be used.

10.2.4 - Tolerance of administration of dl1520

Patients with primary intra-oral squamous carcinoma tolerated the viral injections well. Some pain and swelling at the injection site was noticed but there were no dose limiting toxicity. Most significantly, the study clearly demonstrates that the virus does not harm normal, non-tumour tissue. A significant injection of the virus was given to an area of normal tissue within the mouth of each subject. This did not result in any case of gross damage to this area and we have shown very little virus surviving in these tissues. The virus is therefore well tolerated and safe in this patient group.

10.2.5 - Immune system response to the virus

Our results did indicate that a single injection of the virus did lead to a transient drop in $CD4^+$ lymphocytes following an injection. This effect was noted at the 24hr timepoint and seemed to disappear by 72 hrs. The cause of this is not clear at present, but did not seem to have a deleterious effect on the subjects in the trial. This phenomenon has not previously been noted.

10.2.6 - Suggestions for further work

Dl1520 remains a promising agent for the treatment of SCC of the head and neck. It appears to be safe and well tolerated and in the clinical setting to be specific for tumour tissue as compare to normal tissue. This selective effect is probably due to the action of

the p53 gene in causing apoptosis in normal tissues although the mechanism of action of the virus in p53 wild-type tumour tissues requires further investigation.

Clearly there are many possible avenues for further research using this agent. The virus has already been tested in phase II trials where patients with recurrent SCC of the head and neck were treated with direct intra-tumoural injections of the virus alone or in combination with intra-venous chemotherapy [138, 139]. A logical extension of this is a phase III trial where patients are randomised to receive chemotherapy alone or chemotherapy plus the dl1520 adenovirus. Such a trial is at present underway and once completed should allow conclusions as to the efficacy of this agent in such cases, as compared to standard treatment regimes.

All of the work contained in this thesis has been concerned with direct administration of the virus. One of the key concepts with this attenuated adenovirus is however its purported selectivity of action. This selectivity should in theory allow the virus to be delivered systemically rather than locally. Systemic delivery would allow the treatment of clinically invisible tumour deposits and deposits not accessible to direct injection.

A clinical trial looking at regional delivery of the virus to patients with metastatic liver tumours (emanating from primary colo-rectal cancer) found that the virus could be safely administered via the hepatic artery at a dose of $3X10^{11}$ pfu [140]. The same group also found that intra-venous administration up to the same dose was safe. A clinical trial with patients suffering metastatic deposits of tumour in the lung has also demonstrated the safety and feasibility of intra-venous delivery of the virus [142]. Doses of up to $2X10^{13}$ pfu of adenovirus were used with no dose limiting toxicities.

In the case of SCC of the head and neck, direct intra-tumoural injection of the virus is relatively straightforward as the recurrent tumour deposits usually affect the cervical neck nodes and are therefore amenable to direct injection. The above trials do however

suggest that intravenous delivery would be possible in such cases, and would have the advantage of allowing treatment of clinically non-apparent micro-metastases and allow any distant metastatic lesions present to also receive treatment. In the future a trial of systemic administration of dl1520 to patients suffering with recurrent SCC of the head and neck could be envisaged.

Appendix 1 - Abbreviations

Cdk – cyclin dependent kinase

ISH – in-situ hybridisation

MOI – multiplicity of infection

Mut – mutant

PBS – phosphate buffered solution

PFU – plaque forming units

SCC – squamous cell carcinoma

WCC – white cell count

WT – wild type

Appendix 2

Reagents

Antibodies

FACS analysis

PE –ve	Becton Dickinson.
FITC –ve	Becton Dickinson.
CD2	Becton Dickinson.
CD3	Becton Dickinson.
CD5	Becton Dickinson.
CD8	Becton Dickinson.
CD14	Becton Dickinson.
RO	Becton Dickinson.
CD56	Becton Dickinson.

Immunohistochemistry

ABC reagent - Vectastain ABC kit.

DAB - Vector Laboratories

Hexon - mouse anti-adenovirus monoclonal antibody Ig1k isotype (Chemicon).

Hexon detection - Biogenex Super Sensitive Immunodetection System, Biogenex.

Blocking serum – Horse IgG, Vectastain ABC kit.

Secondary anti-body – biotinylated antibody, Vectastain ABC kit.

Tunel reagent and Converter POD – Boerringer Manheim in-situ cell death detection kit.

Very Intense Purple – Vector Laboratories.

In-situ hybridisation

Anti-biotin/ alkaline phosphatase conjugate – Vector laboratories
Biotinylated adenoviral DNA probe – ENZO diagnostics
NBT/BCIP – Vector laboratories
Post-hybridisation reagent - ENZO diagnostics
Tween 20 – Vector laboratories

Block

Power Block casein solution, Biogenex.

Buffers

Colour development buffer – 100mM Tris, pH 9.5; 100mM NaCl; 50mM MgCl2.
Proteinase K buffer – 50mM Tris, pH 7.5; 5mM EDTA.
Sodium tricitrate buffer (2.94 g sodium tricitrate in 1l distilled water adjusted to pH 6 with HCL).

Enzymes

Protease - Pronase, Meridian diagnostics
Protienase K - Biogenix

Fixatives

4% formaldehyde in PBS.

Sundries

Hydrogen peroxide

0.1% Triton X-100 – Sigma

Stains and histology reagents.

Mayer's hematoxylin - Vector laboratories

Methyl green – Vector Laboratories.

Nuclear fast red – Vector laboratories.

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